The population structure and demography of *Triturus cristatus* in agricultural landscapes of North-West England

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Declaration

I declare that the work presented in this Thesis is my own.

David Orchard

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Abstract

Triturus cristatus is one of Europe's most rapidly declining amphibians and has been the subject of conservation concern in the UK since 1975. Despite its widespread decline and continued threats from development, *T. cristatus* remains widely distributed in the UK countryside. Traditional farming practices, such as the digging of ponds for livestock, created suitable habitats for *T. cristatus* and consequently the species was much more common in the past. Over the last 70 years the nature of farming has fundamentally changed and the modern landscape provides a comparatively degraded habitat for wildlife. The value of farmland for *T. cristatus* in the UK is often overlooked by conservation efforts for the species, even though it is a valuable habitat and essential for providing connectivity between adjacent populations. Much effort is focussed on the small number of Special Areas of Conservation (SACs) or Sites of Special Scientific Interest (SSSIs) but these cover a very small part of the UK landmass. *T. cristatus* has been protected by law in the UK since 1981, and as a result an estimated minimum of £45 million is spent each year to avoid killing or injuring individual newts where populations are affected by development. In contrast land in agricultural production covers 71% of the UK but funding for proactive conservation of the species across this habitat is minimal and very difficult to obtain.

This thesis has investigated the ecology of *T. cristatus* on farmland in North West England. Data were collected from a total of 32 ponds on 11 sites. Population size estimates are presented for eight farm ponds and are compared with those from three non-farmed ponds. Population size varied markedly between ponds and sites, and some farm ponds supported very small numbers of newts. Population estimates fluctuated markedly between years, highlighting the importance of long term studies. Isolated ponds supported relatively large numbers of individuals, and indeed the highest population estimate was recorded in an isolated pond. This demonstrates that isolation in itself is not a limiting factor for population size. In total, 4693 individuals captured during this study were weighed and measured, and the data were used to compare body condition index (BCI) between populations. There was no clear difference between BCI at farmed and non-farmed sites, suggesting that

BCI of *T. cristatus* on farmland was not adversely affected by modern farming practices. There was an inverse relationship between age and body condition.

The age structure of 13 populations was estimated based on skeletochronology of 548 adults. Individuals in the farmed landscape survived to a maximum estimated age of 14 years, only one year less than the maximum age recorded during this study. Twenty individuals were estimated at 12 years or older. Fourteen of these were from farmed and six were from non-farmed populations. This indicates that both the aquatic and terrestrial habitat of the farmed landscape is sufficient to allow newts to fulfil their natural lifespan. The estimated age of sexual maturity for the majority of individuals was 2-3 years. The median estimated age across all populations was 6.5 years for males and seven years for females. It appears that individuals do not breed as soon as they reach sexual maturity and thus remain in the terrestrial habitat for a much longer period of their lives than previously thought. Males always returned to the pond earlier than females of the same age. In both sexes, individuals aged 8 years and over were on average captured approximately three weeks prior to younger individuals.

Whether population isolation has had any measurable effect on *T. cristatus* was investigated using a genetic study of 23 populations on 13 sites. There was no evidence of a loss of genetic diversity through isolation. This study supported the conclusion of other research that dispersal distances for *T. cristatus* can be much greater than reported by capture-mark-recapture (C-M-R) studies. At one of the farmed sites (Moss Shaw Farm), populations just over 1 km apart were assigned similar genetic characteristics, indicating genetic mixing of those populations. This shows that the modern agricultural landscape is still capable of facilitating the dispersal of individuals.

The results of this research demonstrate that the agricultural landscape in the UK can continue to provide a suitable habitat for *T. cristatus*. Efforts to engage with farmers and landowners to enlist their support for the conservation of this species will therefore be worthwhile.

Chapter One: Introduction

1.1 Amphibians and agricultural landscapes

Despite significant changes in the UK countryside over the last 70 years, land in agricultural production still covers 71% of the UK land area, including diverse habitats from upland hill farms to lowland pasture and arable. However, while some farms have retained their historic landscape features such as hedges, woodland and ponds, the last 70 years have also seen increases in overall productivity which often has required fundamental changes in land management. These include more effective land drainage, removal of hedges and ditches, and the use of herbicides, pesticides and fertilizers. In Europe, some intensively managed agricultural landscapes are now unsuitable for amphibians (Zanini et al., 2008). The intensification of farming practices has led to a decline in the number of amphibian breeding ponds (Nicolet et. al., 2007). Many farm ponds no longer serve a useful purpose and many have been filled in, lost due to succession, or become unsuitable for newts due to excessive shading from trees. These changes have affected amphibians as ponds in a late stage of succession are of low value to them (Gent, 2001). Agricultural nutrient runoff has also led to the eutrophication of many farm ponds, further limiting their suitability for amphibians (Nicolet et al., 2007). Increased nutrient levels can, for example, affect the pH of ponds which can have an adverse effect on embryos and larvae (Beebee & Griffiths, 2005). Ponds in England and Wales are now widely degraded, with around 80% being of poor or very poor quality due to intensive land use (Williams et al., 2010). It is widely regarded that habitat loss and alteration due to changes in agricultural practice and land-use has been the overarching factor causing amphibian populations to decline in large parts of the Holarctic (Collins, 2010; Heatwole, 2013; Trochet et al., 2016).

Despite these reductions in habitat quality, England's agricultural landscape still remains an immensely important habitat for amphibians (Boothby et al., 1995). The network of remaining ponds enables amphibians to survive across many parts of the countryside, allowing links with adjacent populations and thus avoiding genetic isolation. The long-term future of amphibians in the countryside however depends on

whether they will be able to survive in this landscape, or whether a combination of habitat loss and other factors will result in their steady decline.

Although found in most ecosystems, amphibians are the vertebrate group with the globally highest proportion of species threatened with extinction (Beebee & Griffiths, 2005). Their dramatic declines (Stuart et al., 2004) have been linked with many factors including habitat loss and fragmentation (Nyström et al., 2007), loss of breeding ponds (Baker & Halliday, 1999), pollution, human exploitation (Nyström et al., 2007), disease and climate change (Beebee & Griffiths, 2005). Five widespread amphibian species are found on farmland in England: *Rana temporaria* (common frog), *Bufo bufo* (common toad), *Lissotriton helveticus* (palmate newt), *Lissotriton vulgaris* (smooth newt) and *Triturus cristatus* (great crested newt).

1.2 *Triturus cristatus*

Triturus cristatus, although still widespread (Figure 1), has suffered from particularly sharp declines in the UK. Despite having been the subject of much research, our knowledge about its ecology remains incomplete. Only two decades ago, *T. cristatus* was considered to be the only crested newt species in Europe, whereas seven species are recognised today (Figure 2). The latest changes have divided the *karelinii* group into *T*. *karelinii,* T*. ivanbureschi*, and *T. anatolicus* (Wielstra et al., 2014). *Triturus cristatus* populations are declining throughout their range (Edgar & Bird, 2006 and Denoël, 2008). Its conservation status is assessed as favourable in only two out of 22 European countries (Luxembourg and Denmark), a fact which has largely been linked to habitat loss (Denoël, 2012). Declines took place despite *T. cristatus* being a flagship species at the European Union level, and one of the rare amphibians to be specially protected under the Habitats Directive (Edgar & Bird, 2005; Jehle et al., 2011). Both monitoring programs and regional assessments have demonstrated significant population decreases, causing this species to be listed in several regional red lists as endangered (Denoel, 2012). This is partly due to the degradation and loss of habitat, which have affected large parts of Western Europe (Hartel et al., 2010). Water quality is also problematic, with 60% of Europe's

freshwater habitats being reported to be in unfavourable conservation status (Temple & Cox, 2009).

In England, long-term studies have shown considerable losses of ponds; Swan and Oldham (1993) demonstrated that up to 90% of ponds have been lost in some areas (median value of 33%). Most losses have occurred since the 1940s. Williams et al., (1999) suggested that 75% of ponds present in 1900 may have been lost by 2000. *T. cristatus* also appears to be particularly vulnerable to changes in land use. A study over 38 years in Northern France recorded a total of 13 amphibian species during the research. Of these, *T. cristatus* was one of two species which experienced sharp declines. This was associated with changes in land use, most prominently the loss of pasture (between 7 and 22%). Declines were also recorded from ponds which remained occupied by other species, suggesting that fish introductions were a further factor in the declines (Arntzen et. al., 2017).

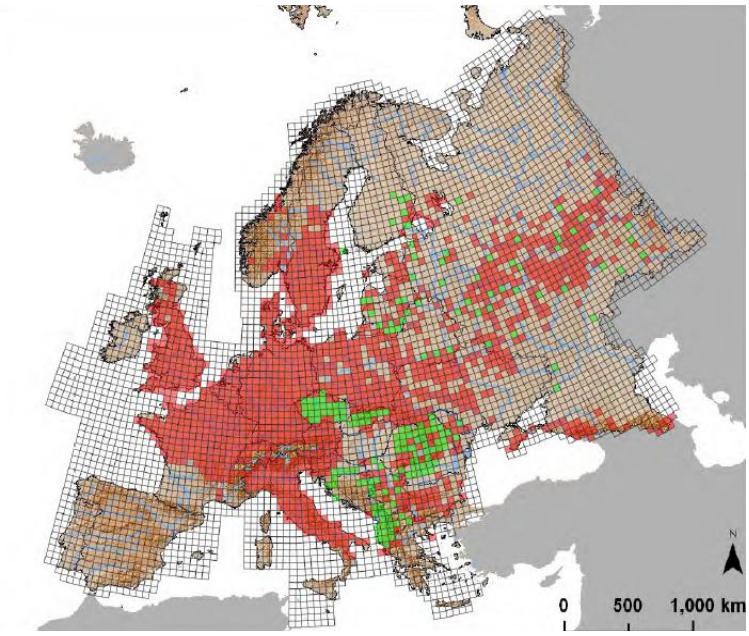


Figure 1. European distribution of the *Triturus cristatus* complex. Colours represent the origin of records. Red corresponds to the national database and green to the *Societas Europaea Herpetologica* (SEH)/ Global Information Facility (GBIF) database (Brown colours represent higher elevations.) Taken from Sillero et al., 2014.

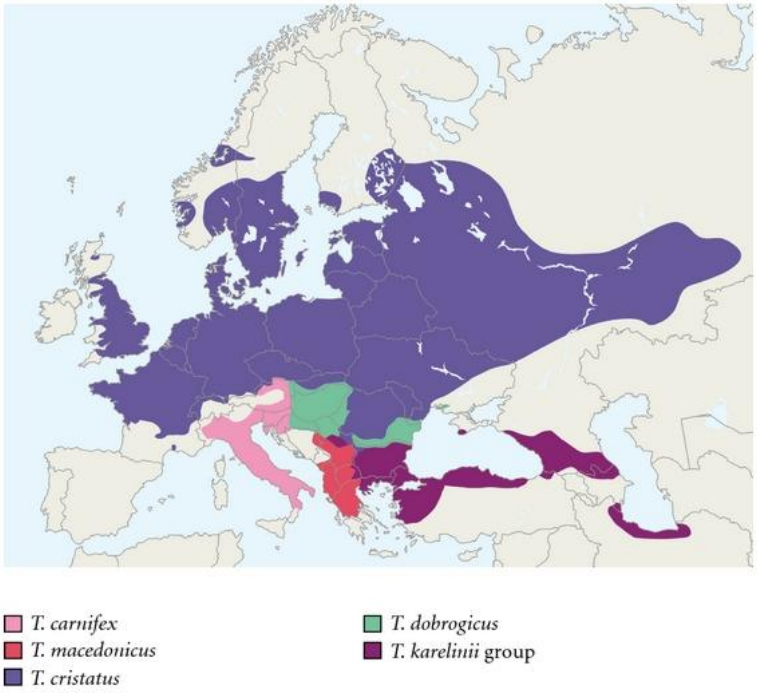


Figure 2: The ranges of the crested newt species showing the approximate distribution of *Triturus carnifex*, *T. macedonicus*, *T. cristatus*, *T. dobrogicus*, and the *T. karelinii* group, taken from Ivanović (2012). Illustrated after Džukić (1993), Kalezić (1997), Wielstra et al., (2010) and Wielstra & Arntzen (2011). Note: the *T. karelinii* group" is now 3 separate species: *T. karelinii*, *T. ivanbureschi* and *T. anatolicus*.

1.3 Distribution of *T. cristatus* in the UK

The population status of *T. cristatus* in the UK has been revised upwards since the early 1990s and it remains a subject for research. Two figures derived in 1993 were often quoted as the estimated number of *T. cristatus* populations in the UK. The Nature Conservancy Council National Amphibian Survey Contract estimated the existence of 17,800 populations (Swan & Oldham, 1993), and the JNCC commissioned review arrived at a similar estimate of 18,300 populations (Langton et al., 1993). Since then, numbers have been revised upwards, with a current estimate of the number of occupied ponds between 50,000 and 100,000 (Wright & Foster, 2009), used by the UK Government in its Article 17 Habitats Directive Report (JNCC, 2013). The most recent estimates show that around *13% of ponds* in England are occupied by *T. cristatus* (http://freshwaterhabitats.org.uk/2015-great-crested-newt-edna-results/). This is similar to estimates from other surveys i.e. Swan & Oldham, 1993, of 11% and Wilkinson & Arnell, 2013, of 12%)

There are two main reasons for the larger 2009 estimate. Survey work during the mid 2000s had revealed high pond occupancy rates by *T. cristatus*. Also, re-assessment of pond numbers nationally suggested a significant increase to the earlier estimates (Biggs et al., 2005). This was particularly important as earlier calculations to determine *T. cristatus* populations had used considerably lower estimates of pond numbers (Wilkinson et al., 2011). Unpublished estimates of total population size vary between 400,000 adults to in excess of three million adults, although the methods used to obtain these figures are questionable (Gent, 2001).

Triturus cristatus can be locally quite common, although throughout much of its range it appears to occur at low population densities (Gent, 2001). In several parts of England, the species is well recorded and remains widespread (Figure 3). These areas include the counties of Lancashire and Cheshire (Grayson et al., 1991 and Guest & Harmer, 2006), Norfolk and Suffolk (Jones, 1988; Buckley, 1989; Langton et al., 2007), Herefordshire and Worcestershire (Hand et al., 2006) and Kent, Surrey and Sussex (Keeble et al., 2009). Nevertheless the local distribution of the species is difficult to ascertain in some areas, due to a shortage of surveys and surveyors,

difficulties over land access and the lack of an effective system to collate survey data. Survey effort has been inconsistent, varying substantially over time and across the UK corresponding to recording projects and surveyor numbers (Gleed-Owen et al., 2005). Conservation efforts have been hampered by this lack of information about distribution and local abundance, and more survey work is needed (Gent, 2001). An improvement to the flow of data from field surveyors to Record Centres would make a significant improvement to the evidence base for *T. cristatus* conservation.

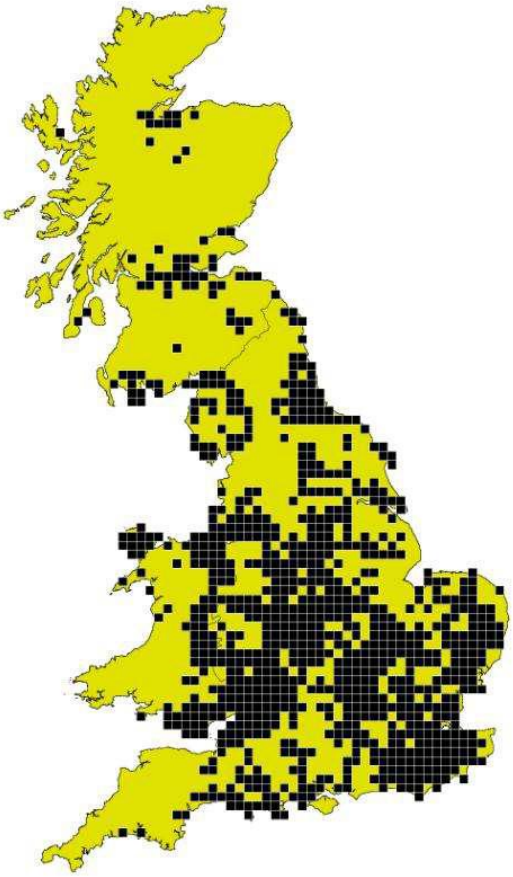


Figure 3: *T. cristatus* occupied 10km squares in Great Britain, based on records including known or suspected introductions.Taken from Wilkinson et al., (2011).

Until recently in the UK, surveys for *T. cristatus* have focussed on the use of four techniques: egg search, torch search, netting and bottle trapping (English Nature, 2001). All require visits in spring when adults return to the pond to breed. The

simplest method is the egg search which involves looking for eggs within folded leaves at the pond edge (Grayson et. al., 1991). Adults are most active within the pond at night, so the torch search uses a high powered torch to look for individuals after dark. Bottle trapping utilises a simple funnel trap made from a cane and a 2 L drink bottle (Griffiths, 1985; Gent & Gibson, 2003) and netting technique involves sweeping a dip net through a pond. All methods have their advantages and disadvantages. Griffiths et al., (1996) calculated that the probability of not finding individuals if present is 1.2% if all four techniques are used. These methods have been widely used since their adoption by English Nature, and subsequently Natural England, as standard survey methods for *T. cristatus* (English Nature, 2001), although other methods have recently become more widely acknowledged. Fish traps (Bock et. al., 2009 and Madden & Jehle, 2013) and Dewsbury Traps (Love, 2013) have both been assessed as being more effective than traditional bottle traps, but are rarely used by consultant ecologists as they are not methods recognised by Natural England. Environmental DNA (eDNA) is a relatively new method of detecting the presence of *T. cristatus* and, due to its approval by Natural England, has gained widespread acceptance since it was first piloted in the UK in 2013. This method is designed to detect mitochondrial DNA that is released from individuals into the environment. Major advantages are that surveys can be more cost effective than those based on traditional methods (Biggs et al., 2015). Also, water samples can be collected in late summer, so eDNA can be used to detect great *T. cristatus* after the optimal survey window for traditional field techniques had passed (Rees et al., 2012). Success rates of 84% and 91.3% have been achieved for eDNA analysis detecting *T. cristatus* ponds (Biggs et al., 2015). Rapid degradation of eDNA in surface water means that only the recent presence of a species can be indicated using this method. In 80 L tank experiments with a toad and a newt species, the longest that eDNA remained detectable after removal of all amphibians was between 9 and 15 days (Thomsen et al., 2012). Recent research shows that for *Triturus cristatus*, eDNA concentrations reach a peak in early June when adult breeding comes to an end, and between mid-July and mid-August corresponding to a peak in larval abundance. eDNA concentration fell rapidly as larvae metamorphosed and left the ponds (Buxton, 2017). One of the striking gaps in this rapidly growing field is the dearth of knowledge about how field and laboratory protocols influence the detection

of eDNA (Goldberg et al., 2011) and how different environmental conditions affect the production, degradation and detection of eDNA. Experiments to systematically compare protocols are urgently needed. As these techniques are refined and developed it is predicted that in the future it will be possible to estimate the abundance of individuals using qPCR-based eDNA analysis (Lodge et al., 2012).

1.4 Habitat preferences of *Triturus cristatus* in the UK

In common with all native British amphibians, *T. cristatus* requires ponds in which to breed and suitable terrestrial habitat in which to forage, rest and overwinter. *Triturus cristatus* has a preference for relatively large, well vegetated ponds which receive direct sunlight for much of the day. Ponds with shallow margins warm up quickly, which enables the rapid development of eggs and larvae. Deep ponds with steep edges are much colder by comparison and are therefore less suitable for the species (Baker et al., 2011). *Triturus cristatus* does not survive well in ponds occupied by fish, nor those completely shaded by trees. Most adults return to ponds to breed in early spring (normally early March) and leave the ponds in the period from the middle of June to the middle of July. The remainder of the year is spent on land, foraging and resting in suitable habitat, dispersing to colonise new ponds in the landscape or overwintering below ground.

Although much is known about the general terrestrial habitat preferences of *T. cristatus*, relatively little is known about how they spend their time on land. This is a major barrier to conservation, since the newts spend approximately two thirds of the year in their terrestrial habitat and systematic insights from laboratory experiments and radio tracking in the field are still frustratingly rare (Jehle et al., 2011). This lack of information was recognised by Oldham et al., (2000) and Cresswell & Whitworth (2004) who recommended that research on newt habitat associations was required to better predict newt density and distribution on the basis of habitat or land use.

Viability modelling has highlighted the importance of focusing conservation efforts and research on the early life cycle stages of *T. cristatus* (Karlsson et al., 2007). The parameters found to be most sensitive for determining population survival over 50 years was fecundity, followed by juvenile survival, adult survival and transition from

juvenile to adult. These factors are therefore crucial for the conservation of the species. Amphibian population dynamics are generally thought to be regulated at the embryonic and larval stages (Vonesh & De la Cruz, 2002). However, sensitivity of juvenile survival has recently been reported for several other amphibian species with different life histories (Conroy and Brook, 2003; Hatfield, 2004). The importance of juvenile survival for the great crested newt is consistent with Sohlman Wiessing (2004), who showed that survival during the first two years was the most sensitive parameter.

In the U.K., *T. cristatus* prefers deciduous woodland, particularly in the vicinity of ponds (Beebee, 1981), shrubs, hedgerows and trees (Jehle & Arntzen, 2000), and scrub and mixed garden habitat (Oldham & Nicholson, 1986; see also Malmgren, 2002). Deciduous woodland is particularly valuable as habitat for over-wintering (Franklin, 1993). Dense ground vegetation cover has also been found to indicate the presence of great crested newt populations (Oldham & Nicholson, 1986). Swan & Oldham (1994) noted that hedges and ditches enhanced the suitability of a site for *T. cristatus.* They found that both landscape features are significant positive determinants of crested newt occurrence in low diversity, improved grassland and arable habitats. Further evidence of their value is provided by Jehle (2000). The occurrence and abundance of newts within pasture is related to the presence and width of uncultivated habitat features (Oldham et al., 2000) but it is unlikely that the absence of these features prevent dispersal across open fields. The affinity of *T. cristatus* for terrestrial habitats with complex structures makes them well suited to brownfield urban or suburban sites, which provide shelter in underground resting places. An example is Orton Brickpits near Peterborough which “contains the largest known population of great crested newt in the UK and possibly in Europe” (JNCC, 2015). In general there is evidence to show that habitat preferences vary according to prevailing landscape characteristics. For example at the edge of its range in the Scottish Highlands, habitat features associated with *T. cristatus* were found to be different from those in its core range in lowland England (Miro et al., 2017). While fish remained negatively associated with the species, organic mud, an important breeding area for potential prey species, was a key feature positively associated with *T. cristatus* presence. Strong links were also shown with mixed *Pinus sylvestris*–

Betula woodland despite sizeable areas of deciduous woodland in the area (Miro et al., 2017).

There are several established methods of investigating habitat preferences of *T. cristatus* on land: pitfall trapping, radio tracking, pit tagging and use of refugia. The most effective method of capturing amphibians on land is amphibian fencing with associated pitfall traps. Information submitted to Natural England under mitigation licence agreements was undertaken by Cresswell & Whitworth (2004). Ninety-eight projects were investigated, but very few trends were discernible regarding habitat preferences of *T. cristatus.* One of their key findings was a strong relationship between the number of captured newts and their proximity to breeding ponds. By far the most captures were recorded within 50 m of ponds, and few animals were captured at distances greater than 100 m, although this finding did not consider habitat type. Peak numbers of adult newts were captured in spring, coinciding with animals captured *en route* to breeding ponds. This may have caused some bias in the study as newts captured on their return to the breeding pond may have overwintered further away. A more detailed review of 44 of these cases did not include sufficiently detailed information to permit a robust analysis (Cresswell & Whitworth, 2004). This study found no information in the licensing files to reveal a significant correlation between habitats (excluding breeding ponds) and capture totals.

1.5 The protection and conservation of *Triturus cristatus* in the UK

Anecdotal evidence for the decline of *T. cristatus* in England was first collected by Beebee (1975), who undertook a questionnaire survey asking for views of naturalists on the status and change in status of the species. While acknowledging the methodological shortfalls (the survey may have considered only 3% of known *T. cristatus* populations in Britain), and the possible bias in sampling, the results showed that the species had declined. Extrapolating the results to a national level indicated that perhaps in excess of 50% of the breeding sites in Britain had been lost between 1966 and 1975 (Gent, 2001). Work by Oldham & Nicholson (1986) indicated that nationally great crested newt sites were being lost at a rate of 2% in 6

years, greater than for other amphibian species. There is evidence of continuing declines. Atkins and Herbert (1996) re-surveyed great crested newt ponds in London and showed a 42% decline in 20 years, and in a re-survey of Hertfordshire identified a 25% decline in 11 years (Atkins & Herbert, 1998).

Similar declines across Europe have led to the European legislation that has resulted in *T. cristatus* becoming one of the most highly protected animals in the UK. It is the only widespread amphibian to receive strict legal protection under Regulation 41 of the Conservation of Habitats and Species Regulations 2010 and Section 9 of the Wildlife and Countryside Act 1981. All individuals, irrespective of life stage, are protected against killing, injury or disturbance. These protection measures still permit development that will damage or destroy *T. cristatus* habitats as long as mitigation and compensation measures are put in place. A key part of the legislation refers to Favourable Conservation Status (FCS). This is defined in the Habitats and Species Directive Article 1(i). One of the key aims of the Directive is to encourage member states to maintain at, or restore to, Favourable Conservation Status species of community interest (Article 2(2)). Conservation status is defined as "the sum of the influences acting on the species concerned that may affect the long term distribution and abundance of its population within the territory." It is assessed as favourable when: "(i) population dynamics data on the species concerned indicate that it is maintaining itself on a long term basis as a viable component of its natural habitats; (ii) the natural range of the species is neither being reduced nor is likely to be reduced for the foreseeable future (iii) there is, or will probably continue to be, a sufficiently large habitat to maintain its populations on a long term basis."

In the UK, conservation objectives have aimed to restore the FCS of the species, but the extent is impossible to quantify due to a lack of records. Despite the strict legal protection for *T. cristatus*, the resulting mitigation work has not always produced the desired outcomes. Lewis et al., (2017) found that a systematic evidence review could not support the notion that mitigation actions result in self-sustaining *T. cristatus* populations. The legislation does nothing to prevent "passive damage" such as the loss of habitat due to neglect, lack of management or natural processes. This protection is likely to change after the UK leaves the EU.

1.6 Conservation action for *Triturus cristatus* in the UK

Agri-environment schemes have attempted to address the impacts of intensive agriculture on farmland habitats with grants aimed at improving the landscape for wildlife. Given the widespread distribution of *T. cristatus* on farmland, providing incentives to farmers to manage their land sensitively for this species would appear to be an effective method of improving *T. cristatus* habitats. In practice this has not happened, as despite its protected status *T. cristatus* is not targeted as a recipient of agri-environment funding. In 2009 approximately £400 million was provided to farmers and land managers in return for them farming in a more environmentally sensitive manner. This was distributed across 58,000 agri-environment scheme agreements covering over 6 million hectares – almost 66% of the agricultural land in England (Natural England, 2009). Restoration of species-rich semi-natural grassland was the single most common option, featuring in 44% of HLS agreements. Payment towards pond creation or management has only been possible if a site was deemed part of a Higher Level Stewardship agreement and the criteria for acceptance into this were very stringent. Higher Level Stewardship was closed in 2016 and has been replaced with Countryside Stewardship. This scheme is even more competitive. Given that *T. cristatus* is not a targeted species, there is even less likelihood that this scheme will provide funding for *T. cristatus* conservation.

The importance of *T. cristatus* breeding ponds as habitats for other species is often overlooked, although small waterbodies can support many aquatic plants and invertebrates. It is estimated that two thirds of Britain's fresh water plant and animal species are found within pond habitats (Williams et al., 2008) and so maintaining a network of high quality ponds has far-reaching benefits, not only for *T. cristatus* but also for many other non-protected species.

1.7 *Triturus cristatus* metapopulations

The study of *T. cristatus* in their natural environment is normally focussed on a population, or cluster of populations. There are at least 16 definitions of population reflecting ecological, evolutionary and statistical models (Waples & Gaggiotti, 2006).

A useful definition in relation to *T. cristatus* is the number of individuals living in sufficiently close proximity that any member of the group can potentially reproduce with any other member (Frankham et al., 2010). In relation to the study of *T. cristatus*, a population is generally accepted as being those individuals using the same breeding pond. Importantly, in cases where several nearby ponds are occupied by *T. cristatus*, individuals will disperse between these ponds. Dispersal can be defined as unidirectional movements from natal sites to breeding sites that are not the pond of birth and not part of the local population. Amphibian migration can be defined as movements, primarily by adults, towards and away from aquatic breeding sites (Semlitsch, 2008; Sinsch, 2014). Populations which are connected by the movement of individuals are therefore not isolated, and the identity of individuals in each pond will change over time. Populations which are connected in this way are known as metapopulations (Hanski et al., 1995), and this population structure applies to many amphibian species.

Metapopulations have been defined as a collection of partially isolated breeding habitat patches connected by occasionally dispersing individuals. Each patch exists with a substantial extinction probability and long-term persistence occurs only at the regional level of the metapopulation (Smith & Green, 2005). The partially isolated populations undergo local extinctions and recolonisations (Hanski & Gaggiotti, 2004). There are various metapopulation concepts ranging from very simple models utilizing a minimum of data (Levins, 1969) to much more complex models which incorporate many environmental variables (Sjogren Gulve & Ray, 1996; Harrison & Taylor 1997; Hanski, 1999). Four conditions have been outlined as necessary to demonstrate the existence of a metapopulation (Hanski & Kuussaari, 1995: Hanski, 1999): 1) habitat patches support local breeding populations, 2) no single population is large enough to ensure long-term survival, 3) patches are not too isolated to prevent recolonization, and 4) local dynamics are sufficiently asynchronous to make simultaneous extinction of all local populations unlikely.

There is a lively debate as to whether typical amphibian breeding ponds represent true metapopulations (Smith & Green, 2005). *Triturus cristatus* is generally assumed to utilise a metapopulation structure, but a study by Jehle et al., (2005) of 15 ponds

in Northern France found that recent migration had only taken place between five population pairs. This mostly took place from large to small populations without any movement in the opposite direction and this supports the idea of a source-sink process within the *T. cristatus* metapopulation system (Jehle et al., 2005). In the source-sink model, the “source” provides all the input to the surrounding “sink” populations. Knowledge of migration rates is vital to the understanding of demographic processes in metapopulations, but the voluminous theory is far from being matched with empirical data, even for the most intensively studied taxa (Bowne & Bowers, 2004). It is extraordinarily difficult and resource intensive to attempt to measure dispersal rates and dispersal distances of *T. cristatus*, which is why genetic studies are so important.

1.8 Population fragmentation and isolation

The metapopulation structure enables the recolonisation of ponds where extinctions have occurred, allowing a species to maintain its range. Changes to either the pond or terrestrial habitat can affect population dynamics, and connectivity between them is essential to maintain a viable population (Crooks & Sanjayan, 2006; Denoël & Ficetola, 2008). High pond density has also been recognised as an important factor linked to *T. cristatus* habitat (Joly et al., 2008). Fragmentation of habitats can prevent individuals moving between nearby ponds, resulting in their isolation and thus disrupting the metapopulation structure (Werner et al., 2007). This can lead to a reduction in the total number of occupied ponds and the genetic isolation of populations. In contrast, the creation of new ponds and management or restoration of existing ponds can reduce the risk of *T. cristatus* disappearing from an area (Karlsson et al., 2007). Climate change and habitat fragmentation have both been cited as reasons to consider the importance of suitable habitat at a landscape level (Carey & Alexander, 2003; Cushman, 2006). Habitat loss and habitat fragmentation usually occur together creating isolated amphibian habitats. This can have dramatic effects on the structure of amphibian populations as well as the condition and health of individuals (Wood et al., 2003). The loss of suitable habitat which enables species to disperse may be one of the important causes of amphibian decline (Marsh &

Trenham, 2001). Conversely, species occurrence has shown a positive association with connectivity (Werner et al., 2007; Zanini et al., 2009). Suitable breeding ponds situated in appropriate places can provide a vital resource for amphibians, not only to reinforce but also to expand the range of existing populations (Kent Reptile and Amphibian Group, 2011). Assessing the long-term viability of *T. cristatus* populations is complicated by a lack of long-term data to determine turnover rates and stability of populations (Werner et al., 2007).

The distance over which amphibians can disperse is a key factor in the survival of a metapopulation. Amphibians have been viewed as animals with poor dispersal abilities (Ficetola & De Bernardi, 2004) and a limited ability to disperse and colonize new ponds (Marsh & Trenham, 2001). A number of studies have linked these characteristics to high genetic differences between amphibian metapopulations (Hitchings & Beebee, 1997; Richardson, 2012). However, assumptions may have underestimated the true travelling distance of amphibians as capture-mark-recapture studies can misjudge dispersal rates (Sinsch, 2014). An extensive literature review (Smith & Green, 2005) found that in 74% of amphibian studies, the assumptions of the metapopulation model were not tested. Those studies that covered larger areas tended to report longer maximum movement distances, which has implications for the design of mark-recapture studies.

1.9 Estimating population dynamic processes in *T. cristatus*

The size of a population can be regarded as a measure of status, but a true understanding of this depends on knowledge of the proportions of each life stage and age structure, as well as total numbers (Oldham et al., 2000). Population size fluctuations can be addressed in a straightforward way with standard field methods, but to assess the exchange of individuals between populations at the landscape scale is difficult with fieldwork alone. The only way to track individuals is using capture-mark-recapture methods (C-M-R) which are extraordinarily labour intensive. This approach has been regarded as the most precise among indirect methods (Caughley, 1977). It is also one of the simplest (Ennos & Bailey, 1995). A method of marking individuals is required so that recaptured animals can be identified. In the

case of *T. cristatus*, this can be done using belly pattern photographs that represent the “marking” of individuals (Lewis, 2012). The black and yellow belly patterns of adult *T. cristatus* are unique and enable individuals to be reliably identified (Hagstrom, 1973). Belly pattern data therefore provides a reliable record of individuals captured and an easy method of counting recaptures (Oldham & Nicholson, 1986). A long term C-M-R study has, for example, been conducted in Kent (Griffiths et al., 2010). A total of 108 bottle traps (Griffiths, 1985) were used per year and each pond was surveyed from the beginning of March to the point at which no more newts were captured, generally mid July. This represents approximately 20 capture visits per year. By 2006 a total of 2647 captures had been identified as 1013 individuals. 2.3% of these individuals were shown to have moved between ponds which were between 200 and 800m apart. However, given the small number of newts which are likely to move between adjacent ponds, it is possible that C-M-R could fail to detect such all such movements. Genetic studies are a much more rigorous method of detecting movement of individuals between ponds. Although there is still scope for error (for example due to small sample sizes or errors in genotyping) it is the best method available. Despite this, temporal population genetic studies are scarce compared to studies on spatial population structure (Jehle et al., 2001).

The use of Body Condition Index (BCI) scores to assess the physical state of an individual has been widely applied in many animal population studies including amphibians (Cooke & Arnold, 2003; Kopecký et al., 2010), seabirds (Lormee et al., 2003), and mammals (Schulte-Hostedde et al., 2001). However, despite its widespread use, the term “body condition” is used to express a range of concepts which can differ substantially between studies. Most authors are usually referring to the relative size of energy stores (Green, 2001) and generally, animals with a high BCI are predicted to be in better condition with greater energy reserves (usually fat) than animals in poorer condition (Dobson 1992 and Schulte-Hostedde et al., 2001). However this may not be true in all individuals, as in females BCI scores may simply reflect the capacity to hold eggs (Halliday & Tejedo, 1995, Malmgren & Thollesson, 1999 and Cooke & Arnold, 2003). Therefore values for BCI in female amphibians are

more likely to indicate their level of fecundity. Also, amphibians hydrate when entering the water which will affect mass and thus BCI scores (Griffiths, 1996).

1.10 Investigating the effect of isolation on the genetic structure of *T. cristatus* populations in the farmed landscape

Population genetics and population size should be considered in conservation biology. This is because low population size, low genetic variability and isolation of populations can all have detrimental effects on populations and their viability, both through demographic and genetic factors (Lande, 1988; Freeman & Herron, 1998). For amphibian protection and management plans, their local population dynamics as well as the degree of population connectivity must be considered (Semlitsch, 2000). However genetic data will only help amphibian conservation when integrated into current and future action plans (Hedrick, 2001; Jehle & Arntzen, 2002). Although many European amphibian species such as *T. cristatus* have suffered serious declines they are not yet at imminent risk of extinction. For these species, genetic studies are particularly valuable. Due to negative effects of inbreeding and genetic drift, small isolated populations are more likely to become extinct over time (e.g., Saccheri et al., 1998). Small isolated populations can also become subject to inbreeding (Beebee, 2005). Population genetic theory states that in small, isolated populations, loss of genetic variability from random genetic drift may reduce future adaptability (Lande, 1988). Small and isolated populations are therefore expected to show lower levels of genetic variability than large populations that belong to a highly connected metapopulation. A high degree of genetic diversity may be required for populations to respond adequately to changing selective pressures, especially in highly dynamic anthropogenic environments. The abundant genetic diversity found in large populations contrasts with that found in many small or bottlenecked populations (Garner et al., 2005). Therefore small, remnant populations of *T. cristatus* which are isolated in the farmed landscape may not be capable of surviving in the long term. Genetic diversity is also important due to a positive correlation with fitness (Reed & Frankham, 2003; Leimu et al., 2006). This problem was observed, for example, in Sweden where small populations of adder, *Vipera berus*, produced a high proportion of deformed and stillborn offspring due to inbreeding (Madsen et al.,

1996). Assessing the genetic diversity of threatened amphibian species can therefore provide clues for the long-term viability of their populations (Schön et al., 2011). Genetic studies on *T. cristatus* have been conducted elsewhere in Europe (Jehle et. al., 2005 and Schön et al., 2011), but not yet specifically in England (although see Jehle et al., 2013 and O'Brien et al., 2015 for a study in Scotland).

A context for genetic studies can be provided by incorporating information about population size, and population estimates can in turn be used in conjunction with genetic data to compare actual with effective population sizes (Jehle et al., 2001). The size of a population is generally taken to be the total number of individuals at a certain locality, but from an evolutionary point of view only those individuals which are successful in reproduction are important. Therefore, the census size of a population is distinguished from the effective population size N_e (Wright, 1931). Current efforts for protecting and sustaining endangered and rare species often focus on the maintenance of genetic diversity (Hedrick & Kalinowski, 2000) and only the effective population size determines the amount of genetic variation maintained over time. Intuitively one might expect the effective population size to be close to the adult population census size, but parameters such as reproductive failures, skewed sex ratios and substantial reproductive skews caused by specific mating systems can bias N_e up to several orders of magnitude below census size (Frankham, 1995; Jehle & Arntzen, 2002).

Viable *T. cristatus* populations depend on the immigration of individuals from neighbouring ponds (Halley et al., 1996) and the movement of only one individual per generation between populations is theoretically sufficient to maintain genetic diversity. Some studies show that only a small number of *T. cristatus* individuals migrate between ponds (Griffiths et al., 2010) but reliance on C-M-R data is likely to underestimate the true extent of dispersal. Field studies about dispersal rates are increasingly supplemented with genetic approaches, but a combination of findings from the field and the laboratory has proved difficult, mainly due to the differing nature of the phenomena studied (migration vs. gene flow). Previously used guidelines for maximum dispersal rates in native amphibians might be too low, and

adjacent breeding sites might be less decoupled than previously thought (Jehle & Sinsch, 2007).

Local genetic diversity of *T. cristatus* is likely to reflect human alterations to natural habitats (Krupa et al., 2002). Amphibians have relatively low dispersal abilities and are often philopatric, leading to distinct populations that can represent unique genetic entities despite geographic proximity (Kimberling et al., 1996, Waldmann & Tocher, 1997 and Driscoll, 1999). The most appropriate method of investigating the extent of isolation of *T. cristatus* populations typical for the farmed landscape is a genetic study using microsatellite markers. Such a study also has the ability to quantify the effects of isolation on a population. Microsatellites occur in high numbers in every eukaryote genome, and consist of tandem repetitive units of DNA typically less than five basepairs in length, with a high variability due to different repeat numbers (Jehle & Arntzen, 2002; Frankham et al., 2010). The ubiquity of microsatellites, along with their high variability, has made them favoured markers for population genetic studies (Garner, 2002). Microsatellites are also indispensable tools for determining patterns of paternity and have proven to be extremely valuable for evaluating gene flow and patterns of interpopulation structure. The only way to detect microsatellites is by using PCR primers. PCR products can be separated by gel electrophoresis, showing their size and allowing the length of the microsatellite to be determined. Microsatellite studies have also been used to infer the effective sizes of *T. cristatus* and *T. marmoratus* populations (Jehle et al., 2001).

The three least invasive methods of collecting genetic material from amphibians are buccal swabbing, skin swabbing and the collection of eggs. Buccal swabbing has been shown to provide enough DNA for microsatellite genotyping in a range of amphibian species (Pidancier et al., 2003; Broquet et al., 2007). However, collecting buccal cells with cotton swabs requires levering open the upper and lower jaw with a sterile spatula which may lead to an amount of bleeding (Pidancier et al., 2003). Some species are easily handled as they tend to keep their jaws opened during sampling, whereas *T. cristatus* usually keep their mouths closed and can be easily injured with either rigid tape or cotton swabs (Prunier et al., 2012). The collection of eggs which have developed to the tailbud stage provides a reliable source of DNA

for genotyping. Freshly laid eggs are not collected as they consist mainly of yolk and contain little genetic material (Jehle et al., 2013).

The main questions addressed by this research are (i) How variable is *T. cristatus* population size on farmed sites and how do these populations compare with those on non farmed sites? (ii) Is there a difference between the age structure of *T. cristatus* populations on farmed and non farmed sites? (iii) Does isolation have any adverse effects on *T. cristatus* populations as measured by the level of genetic variation, and can genetic mixing occur between ponds within the modern farmed landscape?

Chapter Two: Study sites and summary methodology

2.1 General considerations

All of the sites included in this study are located in the North West of England. Seven main study sites were chosen on the basis of local knowledge and contacts. Whilst this did not provide a random sample it nevertheless provided a number of habitats which were reasonably representative of those in which *T. cristatus* is present. The sample included farm ponds surrounded by intensively managed land used for silage production (Marlings), land used less intensiveley as pasture for cattle (Lane Head and Moss Shaw), horses (Moss Shaw and Seddon Fold) and sheep (Wittlestone Head). Data were collected between 2013 and 2016. The seven main study sites included five livestock farms: Lane Head Farm, Marlings Farm, Moss Shaw Farm, Seddon Fold Farm and Wittlestone Head (2.2.4 – 2.2.8) and two sites favourably managed for *T. cristatus* (Bolton Garden pond and Gorse Hill Nature Reserve (2.2.2 – 2.2.3). The favourably managed sites were used for comparison with the farmed sites and referred to as controls. Four subsidiary control sites were added in 2014 and 2015: Acorn Bank, Hic BIbi, Raven Crag and Rixton Claypits (2.2.9 – 2.2.10). The location of sites is shown in Figure 4 and the list of ponds from which data have been collected is shown in Table 1. Population estimates are presented for 11 ponds, age data for 13 ponds and genetic data for 23 ponds. The data collected from these ponds are shown in Table 2.

2.2 Location of study sites

The location of study sites is shown in Figure 4. Table 1 shows all the ponds where data were collected during this study and Table 2 summarises the data which was collected.This is followed by a brief description of each study site. Location maps for all study ponds are shown in Appendix 1 and photographs of the ponds where population sizes were estimated are shown in Appendix 2.

Figure 4: Location of the seven main study sites and the four subsidiary sites (Acorn Bank, Hic Bibi, Raven Crag and Rixton).

Table 1. A list of all the ponds where data have been collected during this study. The pond abbreviations will be used throughout this thesis. The ponds shown in bold are those from which population estimates, age data as well as genetic data has been collected.

Region	Site	Pond name	Abbreviation	Grid reference
Cumbria	Acorn Bank	**Acorn Bank**	AB	NY 61764 28207
Greater Manchester	Moss Shaw	Ainsworth Lodge	AL	SD 77151 09214
	Bolton	**Bolton garden pond**	Bgp	SD 72943 13483
	Moss Shaw	Bradley Fold iris pond	BFi	SD 76434 08513
	Moss Shaw	Bradley Fold Typha pond	BFt	SD 76460 08479
Lancashire	Gorse Hill	**Gorse Hill main pond**	GH	SD 39741 07803
	Gorse Hill	Gorse Hill Jills pond	GHj	SD 39155 07863
	Gorse Hill	Gorse Hill marl pit pond	GHmp	SD 39745 07797
	Hic Bibi	Hic Bibi main pond	HB	SD 56757 12708
	Hic Bibi	Hic Bibi dipping pond	HBd	SD 56830 12692
	Hic Bibi	Hic Bibi shallow pond	HBs	SD 56783 12608
	Lane Head	**Lane Head main pond**	LH	SD 53726 42767
	Lane Head	**Lane Head south**	LHs	SD 54392 42647
	Lane Head	**Lane Head neighbour's pond**	LHn	SD 53849 42808
	Lane Head	Lane Head small deep pond	LHsd	SD 53810 42933
	Lane Head	Lane Head south shady pond	LHss	SD 54119 42778
	Lane Head	Lane Head High House Farm	LHhh	SD 53367 43096
	Marlings	**Marlings main pond**	Marl	SD 59383 36262
	Marlings	**Marlings hedge pond**	Mhp	SD 59260 36544
	Marlings	Marlings dead sheep pond	Mdsp	SD 59222 36051
	Marlings	Marlings garden pond	Mgp	SD 59507 36847
	Marlings	Marlings horsetail pond	Mhtp	SD 59128 35977
	Marlings	Marlings Redmaine pond	MR	SD 59941 36026
Greater Manchester	Moss Shaw	**Moss Shaw main pond**	MS	SD 76775 08712
	Moss Shaw	Moss Shaw car park pond	MScp	SD 76834 08601
	Moss Shaw	Moss Shaw muddy pond	MSmud	SD 77043 09068
	Moss Shaw	Moss Shaw spearwort pond	MSsp	SD 77041 09161
Cumbria	Raven Crag	Raven Crag	RC	NY 46607 29327
Greater Manchester	Seddon Fold	**Seddon Fold Farm**	SF	SD 67940 07427
Cheshire	Rixton Claypits	Rixton Claypits	R	SJ 68519 90135
Lancashire	Wittlestone Head	**Wittlestone Head main pond**	WH	SD 71951 19333
	Wittlestone Head	Wittleston Ramwells pond	WHR	SD 71964 19004

Table 2. Data collected during fieldwork. C-M-R = capture-mark-recapture study undertaken at these ponds. Eggs = eggs collected from these ponds for the genetic study. Weight and Length = individuals weighed and measured. Toe = toes clipped for skeletochronology. Y = population estimates calculated at these ponds. (Y) = ponds surveyed but no population estimates calculated due to the very small number of captures.

Pond name	Abbreviation	2012	2013			2014				
		CMR	CMR	eggs	weight	CMR	eggs	weight	length	toe
Acorn Bank	AB									
Ainsworth Lodge	AL						Y			
Bolton garden pond	Bgp	Y	Y	Y	Y	Y	Y	Y	Y	Y
Bradley Fold iris pond	BFt						Y			
Bradley Fold Typha pond	BFi						Y			
Gorse Hill main pond	GH		Y	Y	Y	Y	Y	Y	Y	Y
Gorse Hill marl pit pond	GHm		(Y)							
Gorse Hill Jills pond	GHj			Y			Y			
Hic Bibi dipping pond	HB						Y			
Hic Bibi main pond	HBd						Y			Y
Hic Bibi shallow pond	HBs						Y			
Lane Head south	LHs		Y	Y	Y	Y	Y	Y	Y	Y
Lane Head High House Farm	LHhh						(Y)			
Lane Head main pond	LH		Y	Y	Y	Y	Y	Y	Y	Y
Lane Head neighbours pond	NHn		Y				Y			
Lane Head small deep pond	LHsd					(Y)	Y			
Lane Head south shady pond	LHss						Y			
Marlings dead sheep pond	Mdsp		(Y)	Y	Y	Y	Y	Y	Y	
Marlings garden pond	Mgp		(Y)	Y	Y		Y			
Marlings hedge pond	Mhp		Y	Y	Y	Y	Y	Y	Y	
Marlings horsetail pond	Mhtp		(Y)							
Marlings main pond	Marl		Y	Y	Y	Y	Y	Y	Y	Y
Marlings Redmaine pond	MR						Y			
Moss Shaw car park pond	MScp		(Y)							

Moss Shaw main pond	MS	Y	Y	Y	Y	Y	Y	Y	Y
Moss Shaw muddy pond	MSmud				(Y)	Y	Y	Y	
Moss Shaw spearwort pond	MSsp				Y		Y	Y	
Raven Crag	RC						Y	Y	Y
Rixton Claypits	R					Y	Y	Y	Y
Seddon Fold	SF	Y		Y	Y		Y	Y	Y
Wittlestone Head	WH				Y	Y	Y	Y	Y
Wittlestone Head Ramwells pond	WHR					Y			

Table 2 (continued)

Pond name	2015				2016		
	CMR	weight	length	toe	CMR	weight	length
AB	Y	Y	Y	Y			
AL							
Bgp	Y	Y	Y	Y	Y	Y	Y
BFt							
BFi							
GH	Y	Y	Y	Y	Y	Y	Y
GHj							
HB							
HBd							
HBs							
LHs							
LH	Y	Y	Y	Y	Y	Y	Y
NHn	Y	Y	Y	Y	Y	Y	Y
LHsd							
LHss							
Mdsp							
Mgp							
Mhp	Y	Y	Y	Y			
Mhtp	Y	Y	Y	Y			
Marl							
MS	Y	Y	Y	Y			
MSmud							
MSsp							
RC							
R							
SF	Y	Y	Y	Y	Y	Y	Y
WH	Y	Y	Y	Y	Y	Y	Y
WHR							

2.2.1 Acorn Bank (east of Penrith, Cumbria)

Acorn Bank is a country house which has been owned by the National Trust since 1950. It has 72 ha of parkland, much of which is sheep grazed pasture and woodland. Only one of the three ponds on the site is occupied by *T. cristatus*, an ornamental pond approximately 4m in diameter. The other two ponds contain fish, and reportedly have done so for many years. There is no specific management for *T. cristatus*.

2.2.2 Garden pond (Bolton, Greater Manchester)

This is a relatively large suburban garden (30x15 m) with four ponds, two fairly large (10x2 m and 7x2 m) and two small (1.2x1.2 m). Due to the proximity of the ponds (maximum 2m apart), all four were treated as supporting a single population and data from all ponds was combined. Approximately half the garden is managed for wildlife and includes dry stone walls, log piles and a large compost heap. A total of forty adults were introduced here in 1992-93 using individuals from two separate populations in the Greater Manchester area. No further introductions took place and the population is assumed to have remained isolated.

2.2.3 Gorse Hill Nature Reserve (Aughton, near Ormskirk, Lancashire)

This is a private nature reserve established in 1996 and open to the public. The site includes several wooded areas and meadows together with three ponds, one of which is very small (5 x 2 m). All three ponds were surveyed in 2013 but adult *T. cristatus* were only recorded in one pond known as Seldom pond.

2.2.4 Lane Head Farm (Claughton-on-Brock, Lancashire)

This is a tenanted farm, managed for beef and dairy cattle. Approximately half the fields are grazed during the spring and summer and the other half are used for silage production. All the land is fertilized with slurry produced by the herd of 50 cows and NPK fertilizer is applied in the spring. Until May 2013 the farm had a total of 6 ponds,

two of which were confirmed as breeding ponds for *T. cristatus*. In June 2013, 5 new ponds were created and one managed as part of a conservation project. These were checked for presence of *T. cristatus* and one of the ponds within 200m of the main study pond was colonised in 2014. This pond was surveyed a number of times in 2014 and 2015 but only one female *T. cristatus* was captured.

2.2.5 Marlings (Longridge, Lancashire)

This is a privately owned farm managed primarily for silage production with sheep grazing in winter. Prior to 2004 the farm was managed by the owner as a dairy farm but since his retirement it has been managed by two tenant farmers. Marlings includes 7 ponds and a small garden pond, five of which were confirmed as breeding ponds for *T. cristatus* in 2011. This appeared to be the most intensively managed of all the study sites.

2.2.6 Moss Shaw Farm (Bury, Greater Manchester)

This is a tenanted farm on the edge of suburban Bury. It is largely grazed by beef cattle but approximately one third is intensively grazed by horses. The farm includes a total of five ponds, four of which have been confirmed as *T. cristatus* breeding ponds. One of these is a temporary pool and another is shallow and devoid of macrophyte vegetation.

2.2.7 Seddon Fold Farm (Westhoughton, Greater Manchester)

This is a tenanted farm, most of which is intensively grazed by horses but with some grazing from beef cattle. The farm includes a total of five ponds, only one of which contains *T. cristatus.* Forty adults were introduced into this pond in consecutive years 1988-89 and no further introductions took place. Two of the other ponds contain large populations of fish and two are almost completely full of silt and vegetation.

2.2.8 Wittlestone Head (Darwen, Lancashire)

This is a tenanted upland farm grazed by sheep. It is within 30 m of excellent terrestrial habitat for *T. cristatus* provided by a wooded railway cutting. The farm includes a single pond and the nearest pond, which also supports *T. cristatus*, is approximately 400m away on adjacent land.

2.2.9 Rixton Claypits (Warrington, Cheshire)

This site supports Cheshire's largest known *T. cristatus* breeding population and is designated a SSSI and SAC for the species. The habitat has developed within an extensive disused brickworks site excavated in glacial boulder clay. Excavations have left a series of hollows which have filled with water since workings ceased in the 1960s, leading to a variety of pond sizes. *T. cristatus* is known to occur in at least 20 ponds across the site. Detailed torch counts have been carried out for over 10 years by the Ranger Service, confirming a minimum population of many hundreds of individuals.

2.2.10 Hic Bibi Nature Reserve (near Chorley, Lancashire)

This is a Local Nature Reserve which has developed by a process of natural succession on a former clay quarry and brick works. It includes seven ponds, three main *T. cristatus* breeding ponds and further three created in 2007 which are in the early stages of colonisation by the species. All the ponds are relatively close to each other (within 100m).

2.2.11 Raven Crag (west of Penrith)

This is a former clay extraction site where surveys have confirmed a large *T. cristatus* population in a deep quarry pond. In 2014, the site was the subject of a development licence application to Natural England following a proposal to construct a holiday village on the site. Many *T. cristatus* individuals have now been moved to a receptor pond just outside the site boundary.

2.3 Summary of data collected

Prior to the collection of any data for this study a licence was obtained from Natural England. This permitted the capture and handling of *Triturus cristatus*, the collection of eggs for the DNA study and the removal of toes for skeletochronology. This licence was renewed each year. Data were collected from 2013 to 2016, with the exception of the garden pond which was also visited in 2012 as a pilot study. A total of 5021 individuals were captured over the four years of this study. The belly pattern of each one was photographed (in order to calculate population estimates), a toe was removed from 548 individuals (a maximum of 50 adults per pond to be used in a skeletochronology study) and the weight and snout/vent length (SVL) of individuals captured from 2014 until 2016 was measured in order to calculate body condition. Eggs at the tailbud stage were collected from the ten main study ponds and some surrounding ponds as the basis for the genetic study to investigate the relatedness of newts in adjacent ponds. In total, data was collected from a total of 32 ponds on 11 sites. Table 1 shows a list of these ponds and an abbreviation for the pond name which has been used throughout this thesis. At sites where more than one pond is present, the abbreviation for the main pond is the same as the site name (e.g. LH refers to Lane Head Farm and its main pond).

The *Triturus cristatus* population in the garden pond cannot be considered as representative of those in the wider landscape due to the different factors acting upon the habitat. These included nearby roads and gulley pots and human disturbance of neighbouring gardens, all of which had the potential to cause an increased risk of mortality of individual newts. Nevertheless the garden pond enabled a detailed study to be undertaken which indicated the effectiveness of research methods, particularly capture-mark-recapture (C-M-R).

In order to assess the quality of aquatic habitats, pond invertebrates were sampled and aquatic and marginal plants were recorded. Visits were done in August 2013 using the PSYM methodology (Pond Conservation, 2002). In summary this method included (i) Three minute sampling of invertebrates using a hand net at each different mesohabitat around the pond edge. (ii) Compiling a list of all the aquatic

and marginal plants found in and around the pond. A summary of the invertebrate families and plant species found in each of the ponds sampled is shown in Appendix 3 and 4. Sampling was done with the assistance of experienced invertebrate ecologist Dr. Jim Fairclough, who identified the invertebrates and plants from as many of the study ponds as possible. A total of 13 ponds at three sites were visited (Marlings, Lane Head and Moss Shaw). Due to limitations of time the remaining three sites (the garden pond, Gorse Hill and Seddon Fold Farm) were not visited. Wittlestone Head was omitted as it was not included in this study until 2014. The results of the plant and invertebrate surveys were sent to The Freshwater Habitats Trust for PSYM analysis. The score is obtained by comparing the data collected with their dataset of ponds which has been collected over many years. The PSYM score provides an assessment of the ecological quality of a pond. The PSYM scores are shown in Table 3.

Table 3: PSYM scores for ponds at which plant and invertebrate surveys were undertaken in 2013. The number plant species and invertebrate groups varied between ponds but all were classified as being either medium or poor quality ponds.

M = medium quality; **P** = poor quality pond

Pond reference	**LH**	**LHs**	**LHss**	**LHn**	**LHhh**	**Marl**	**Mhp**	**Mdsp**	**Mhtp**	**MS**	**MShtp**	**MScpp**
Total number of invertebrate groups	16	13	7	11	10	16	17	7	14	15	13	14
Total number of submerged and aquatic plant species	11	20	11	10	11	11	9	4	9	12	14	18
PSYM score	**M**	**M**	**M**	**M**	**P**	**M**	**M**	**P**	**P**	**M**	**M**	**M**

The number of invertebrate groups shown for each family is the number of individuals collected in each sampling session.

Table 4: Dates on which PSYM samples were collected.

Dates on which samples were collected:			
1	LH: collected 3.8.13	7	Mhp: collected 3.8.13
2	LHe: collected 3.8.13	8	Mds: collected 3
3	LHss: collected 3.8.13	9	Mht: collected 3.8.13
4	LHn: collected 3.8.13	10	MS: collected 24.8.13
5	LHhh: collected 3.8.13	11	MSht: collected 24.8.13
6	Marl: collected 3.8.13	12	MScpp (collected 24.8.13

Note: No *T. cristatus* data was collected from MSht (a pond at Moss Shaw Farm) therefore it is not included in Tables 1 and 2.

Chapter Three: Population size of *Triturus cristatus* in the farmed landscape

Summary

This is the first study in the UK to compare *Triturus cristatus* population sizes in the farmed landscape with those on favourably managed sites. Adults were captured with aquatic traps and identified using belly pattern photographs. Initial surveys indicated that many of the occupied ponds on farmland supported very small populations. Detailed capture-mark recapture estimates from 11 selected ponds for up to 4 years (2013-2016) revealed populations comprising of 12 to 752 adults exhibiting site-dependent population fluctuations between years (increases and declines). Estimated return rates between years were high in a population for which detailed data were available. Deviations from 1:1 sex ratios were a common occurrence, and consistent across years for given ponds. The finding suggests that farm ponds can support *T. cristatus* populations when the key environmental requirements are met.

3.1 Introduction

Understanding the status of *Triturus cristatus* in the farmed landscape is based almost entirely on their presence or absence at a pond. Studies which estimate the true size of a *T. cristatus* population involve considerable investment of time and effort, and consequently such studies are rarely undertaken. A number of studies have been conducted across Europe (Table 5) but only two have been published in the UK: Baker (1999) and Arntzen et al., (1999). This contrasts with the large number of population size class assessments which are frequently conducted by consultant ecologists in the UK. These use a standardised methodology which was devised by English Nature (2001) to produce a simple index that quantifies populations as small (1-9), medium (10-99) or large (over 100 individuals) according to the maximum number of newts seen or captured on one occasion using a standardised methodology.

Table 5: Population estimates for adult *Triturus cristatus* based on C-M-R.

Study site	Mean Popn size	Min-Max	Study years	Reference
UK, Buckinghamshire	113 +/- 59	67-242	8	Baker (1999)
UK, Leicestershire	1 408 +/- 73	1356-1459	2	Arntzen et al., (1999)
Western France, Pas-de-Calais	146 +/- 116	16-346	6	Arntzen & Teunis (1993)
Eatsern France, Bresse	322 _/- 159	209-434	2	Miaud et al., (1993)
Southwestern Sweden, Goteborg	342 +/- 104	230-500	5	Hagstrom (1979)
Northeast Germany, Barnim	34 +/- 25	4-66	7	Stoefer & Schneeweiss (2001
Northeast Germany, Barnim	136 +/- 86	61-305	7	Stoefer & Schneeweiss (2001
Northeast Germany, Barnim	36 +/- 25	6-65	7	Stoefer & Schneeweiss (2001
Northeast Germany, Barnim	53 +/- 34	20-107	7	Stoefer & Schneeweiss (2001
Northwest Germany, Munsterland	101 +/- 15	89-108	4	Glandt (1982)
Western Germany, Siegburg	29 +/- 6	25-33	2	Wenzel et al., (1995)
Western Germany, Kottenforst	125 +/- 64	65-209	4	Blab & Blab (1981)
Western Germany, Drachenfesler Landchen	60 +/- 29	26-97	7	Kupfer & Kneitz (2000)
Western Germany, Drachenfesler Landchen	157 +/- 34	120-186	3	Ortmann et al., (2005)
Eastern Germany, Merseburg	1 129 +/- 146	1 026-1 232	2	Meyer & Grosse (2007)
Eastern Germany, Merseburg	230 +/- 35	205-254	2	Meyer & Grosse (2007)
Eastern Germany, Merseburg	1 229 +/- 919	579-1 879	2	Meyer & Grosse (2007)
Southeast Sweden	357 +/- 133		1	Karlsson et al., (2007)
Southeast Sweden	344 +/- 74		1	Karlsson et al., (2007)
Southeast Sweden	163 +/- 141		1	Karlsson et al., (2007)
Southeast Sweden	32 +/- 54		1	Karlsson et al., (2007)
Southeast Sweden	14+/- 19		1	Karlsson et al., (2007)
Southeast Sweden	5 +/- 13		1	Karlsson et al.,

			(2007)
Southeast Sweden	10 +/- 24	1	Karlsson et al., (2007)
Southeast Sweden	314 +/- 182	1	Karlsson et al., (2007)
Southeast Sweden	92 +/- 52	1	Karlsson et al., (2007)
Southeast Sweden	187 +/- 86	1	Karlsson et al., (2007)
Southeast Sweden	402 +/- 217	1	Karlsson et al., (2007)
Southeast Sweden	99 +/- 61	1	Karlsson et al., (2007)
Southeast Sweden	67 +/- 44	1	Karlsson et al., (2007)

However, a population size class assessment does not give a true estimate of population size and it does not involve recapturing individuals. Instead its purpose is to form the basis of licence applications to Natural England to permit mitigation measures in cases where populations are affected by development. Such licence applications are required as a legal requirement under The Conservation of Habitats and Species Regulations 2010 (as amended) which implements the EC Directive 92/43/EEC in the United Kingdom.

Triturus cristatus population size is likely to depend on factors such as the condition of the breeding pond and terrestrial habitat. However, studies show that population size is typically 20-200 adults and that populations in excess of 1000 individuals are rare (Jehle et al., 2011). However, ecologists often target intermediate to large populations for study and those which are small or very large are not favoured for investigation (Jehle et al., 2011). *Triturus cristatus* populations can remain stable for a number of years (Blab & Blab, 1981) or they may fluctuate significantly over successive years (Hagström, 1979; Hedlund, 1990; Arntzen & Teunis, 1993; Kupfer & Kneitz, 2000; Jehle et al., 2011). Therefore, the results of one year of study can be misleading. The lack of data on *T. cristatus* populations in the UK reiterates the need for studies that are long-term in nature. Few studies have been conducted over consecutive years but these are required to provide a true picture of population size (Pechmann et al., 1991). The longest running and perhaps best known study in the UK has been conducted at the Durrell Institute of Conservation and Ecology (DICE)

in Kent. Four ponds have been studied since 1994: two semi-natural ponds, a disused swimming pool and three garden ponds in close proximity to each other. Population size has varied significantly over this period, the peak estimate in one of the ponds being just over 200 adults (Griffiths et al., 2010). Four small ponds were added in 1998, and a further four were added in 2009. Captures in these new ponds rose steadily to 40 different individuals in 2010 (Lewis, 2012). Another long-term study, conducted by Baker (1999) involved the monitoring of a single pond from1988-1995 and over the eight year period population size ranged from 67-242 adults. After six years with little recruitment, the population increased more than three-fold over two years, probably due to a population crash in the predatory three-spined stickleback (*Gasterosteus aculeatus*). Another study by Arntzen et al., (1999) in Leicestershire found that population estimates at a pond in two consecutive years remained very similar (1356 adults in 1990 and 1471 (+/-75) in 1991). This is one of the largest published population estimates in Europe but it contrasts with the results of another study on the Danube River floodplain in Romania which found that the entire population of the four ponds approximately doubled between 1987 and 1988. This increase was mainly due to recruitment of newts that reproduced for the first time, but older adults were also recruited (Miaud & Cogalniceanu, 2003). Unpublished population studies in the UK include PhD theses by Jarvis (2012) and McNeill (2010).

For the purposes of this study it has been assumed that all *T. cristatus* individuals captured in one pond are part of a discreet population. This assumption has been necessary to calculate a population estimate for each pond, but it is recognised that a small number of individuals may have dispersed, particularly between years. Another factor which is likely to affect the extent to which the population estimate is truly representative is uncertainty over the proportion of individuals that return to the pond each year. These estimates assume that all individuals return each year and that they remain available for capture throughout the breeding season.

3.1.1 Research objectives

This study had four objectives; (i) to estimate population sizes at farmed and non-farmed sites, (ii) to quantify the extent of any size fluctuations between years (iii) to investigate survival and detectability and (iv) to estimate operational sex ratios. The focus of this study was 11 ponds, 8 within the farmed landscape and 3 on favourably managed sites. Population estimates were determined using C-M-R data. Each captured individual was weighed and measured so that a value for body condition index (BCI) could be calculated. Information on BCI is covered in Chapter 4.

3.2 Methods

3.2.1 Fieldwork

Ponds were visited from the time *T. cristatus* started returning to ponds to breed (March) and the time they left the pond (June or July). Data were collected from the garden pond in Bolton in 2012 to test methodologies, with the majority of data being collected in 2013, 2014 and 2015. The aim was to capture as many newts as possible during each site visit and therefore there was no need to follow the Natural England survey methodology (English Nature, 2001). Both Dewsbury traps (Dewsbury, 2011) and fish traps were used, as they have been shown to be more effective than conventional bottle traps (Madden & Jehle, 2013). All survey work was done under NE licence number 20131092, 20140037, 2015-9912-SCI-SCI and 2016-22714-CLS-CLS.

The fish traps (Figure 5) used were collapsible boxes made from nylon mesh. Dimensions were 400 x 200 x 200 mm with a 40 mm diameter hole at both ends to allow newts to enter. The benefits of using these traps was that they were collapsible and easy to transport (up to 60 could be carried by one person). This method was an ethically safer method of catching newts than bottle trapping due to a reduced risk of drowning and it was also more efficient in that it captured more newts (Madden & Jehle, 2013). Dewsbury traps are significantly more bulky and inconvenient to transport than fish traps and over 10 times more expensive (Dewsbury traps for this

study now cost £30 – Figures 6 and 7). They consist of two main parts: a plastic sandwich box with a hole cut out to allow amphibians to enter, and a bin bag which is attached to the box with elastic in place of a lid. A float (made from expanded polystyrene) is inserted into the bag to keep it afloat, providing an air space allowing the newts to breathe. The two capture methods were different from each other in that they were able to catch newts at different depths.

Figure 5: A collapsible fish trap. The white elastic was fitted to create the funnel at either end of the trap. The large zip allows the trap to be opened and the newts to be removed. (The small zip allows fish bait to be held - not required for this study.)

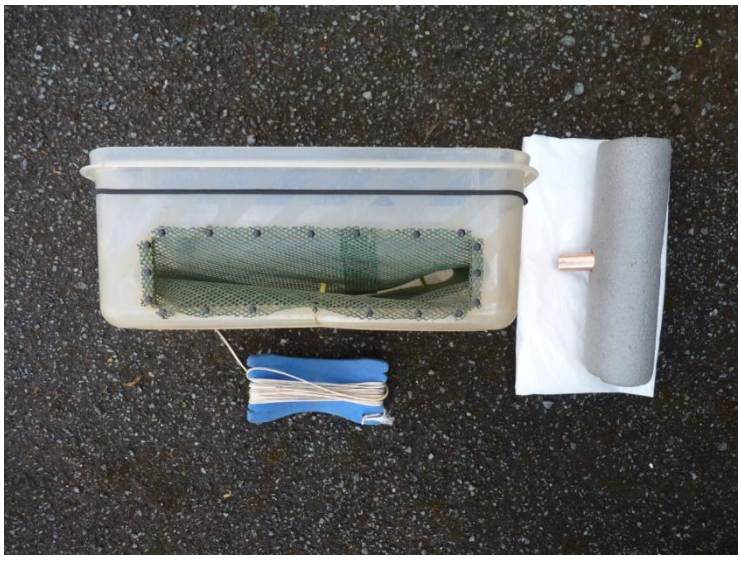


Figure 6: The constituent parts of a Dewsbury trap, including the modified sandwich box with hole to allow access for newts, a plastic bag, polystyrene float with pipe to facilitate exchange of oxygen, elastic to attach the bag to the box and string to allow retrieval of the trap.

Figure 7: The Dewsbury trap assembled and ready to be deployed into a pond.

In accordance with good practice (ARG UK, 2008), all equipment which had been in contact with pond water was sterilised using a solution of Virkon before being used again. This included all fish traps and Dewsbury traps, waders and the glass plate used when photographing newts. Equipment was rinsed in clean water and left in the open air to dry.

Although *T. cristatus* was known to be present at all of the study sites, at the start of 2013 it was unclear how many ponds actually supported the species as no detailed survey information was available. The first priority in 2013 was to survey all ponds at each study site to confirm which ones supported *T. cristatus*. In 2014 a maximum of two ponds per visit were surveyed due to the time taken to photograph and measure each newt. This was approximately double that of the previous year due to the need to obtain samples for the study into age structure. In 2014 capture effort was focussed on one pond at four study sites, and two ponds at the remaining two sites. An additional study pond, at Wittlestone Head, was added in 2014 to increase the

sample size but this had the side-effect of reducing the number of capture visits per site. Traps were deployed at study ponds in late afternoon or early evening. The number used varied depending on the size of the pond (Table 6) from a minimum of 30 at Bgp to a maximum of 130 at Marl and Mhp. (All the ponds at LH were trapped on the same visit as they were on the same site, as were Marl and Mhp).

Table 6: The number of fish traps used per capture visit.

Pond	Number of fish traps used
Bgp	30
GH	90
LH	90
LHn	10
LHs	40
Marl	80
Mhp	50
MS	80
SF	30
WH	60

Fish traps were set at the pond edge with the top of the trap visible above the water to allow amphibians to breathe. Traps were tied to a cane to avoid the possibility of them being lost within the pond. Due to the time taken to set numerous traps (often taking 5-6 hours including travelling time), it was decided to tie three traps to one cane so that traps could be deployed more quickly. This did not significantly affect the distribution of traps around the pond edge due to the large number of traps used. Fish traps were set along the accessible edges of the pond, generally 0.6 m apart. Dewsbury traps were set 2-4 m from the pond edge and attached to a cane set into the bank so that they could be retrieved easily. The box of the Dewsbury trap generally settled 0.4-0.5 m below the water surface, depending on the amount of vegetation present and the depth of the pond. All traps were retrieved the following morning and any *T. cristatus* captured placed in a box. The number of visits made to each pond is shown in Table 7 and the date of capture visits is shown in Appendix 5. Capture visits were undertaken between March and June. All individuals younger than adults were classified as juveniles. This removed any lack of clarity as to

whether young newts should be recorded as metamorphs (having recently emerged from a pond), juveniles (small newts in the year of metamorphosis) or sub-adults (young newts at least one year old but not yet in breeding condition). Given the small number of individuals captured prior to adulthood it was not practical to divide young newts between three age categories for analysis.

During 2014 it became apparent that newts were probably escaping from the fish traps overnight (individuals had up to 8-9 hrs to escape). This was supported by evidence from night torching, where relatively large numbers of newts were seen but small numbers retrieved in the morning. Also, traps were seen containing newts by torchlight but in the morning they were empty. The capture technique was therefore modified in 2015 in two ways. Firstly, night torching was carried out and where possible newts were netted. This was particularly effective at Gorse Hill and the Garden Pond as the water was clear and with shallow edges. It was much less effective at SF and Marl, where the water was deep and turbid. Secondly, traps were checked after nightfall and individuals removed before they had chance to escape. This was very effective at all sites. Traps were generally checked twice, which took between 3 and 4 hrs.

The lowest number of survey visits was conducted in 2014, largely due to the additional time required to toe clip individuals. In 2013 traps were removed from the first pond and the captured newts photographed and weighed before moving to the next pond. This meant that traps remained in the second pond for an extra 4-5 hours, giving individuals longer to escape. At Marl and LH, where two ponds were trapped, low capture results were noticed in the second pond visited. This suggested that newts had escaped before the traps had been removed. From 2014 onwards, all traps were removed from both ponds early in the day and before any newts were photographed. Individuals from the first pond were placed in a container of water and retained until newts from the second had been retrieved, photographed, weighed and measured. A return journey to the first pond was then required to photograph those newts. This took longer, but it reduced the risk of newts escaping before they could be photographed weighed and measured.

A pilot study was carried out at the garden pond in 2012, during which it was found that individuals of *T. cristatus* can be difficult to photograph. Although some remain still for 30 seconds or more, others are immediately keen to escape and move quickly. Newts are unsettled when turned upside down and immediately attempt to turn themselves the right way up. Any belly pattern photographs are difficult to take and require a careful and well practised technique. A new technique was developed which allowed very clear photos to be taken which were completely free from distortion. By trial and error it was found that the simplest and most accurate method of taking a belly pattern photograph was to place the newt on a flat transparent surface, hold it approximately 900 mm above the ground and then take the photograph from below. A piece of glass 200x150 mm, fixed to a timber frame to provide rigidity, was used (Figure 8).

Figure 8: Glass fitted to a frame on which newts were placed before photographing them from below.

When taking the belly pattern photographs it was essential that each one was clear and easy to compare with others. Newts do not remain still for long and it is impossible to measure a moving newt accurately. They often bend, making accurate measurements impossible, and can contract their bodies when stressed. The aim was to obtain a photograph of a newt lying at rest on the flat surface, not twisted or stretching. This would enable straightforward comparison of images thus maximising

the chance of matching up photographs of the same newt taken on different occasions.

Figure 9: Belly pattern photographs taken using different methods. The traditional method used by Lewis (2012) is on the left, the method used for this study is on the right.

In 2013 individuals were photographed and weighed. In 2014 and 2015 they were also measured and toe clips were taken from some newts. In 2016 captures were photographed, weighed and measured. All other amphibians were released immediately as they did not need to be photographed. Each newt was weighed using a digital scale with an accuracy of 0.01 g. Wind had a significant effect on the digital scale, so it had to be used in a sheltered, wind free environment. In 2014, two scales were used to confirm that correct weights were being recorded. Amphibians can substantially lose mass due to water loss in a dry environment (Jehle & Hodl, 1998) therefore newts were kept in a container of water between the time of capture and time of weighing. All individuals were measured from the tip of the nose to the tip of the cloaca, referred to as the snout-vent length (SVL). The most accurate method of doing this was immediately before taking the belly pattern photograph. A ruler was placed against the underside of the glass on which the newt was resting and when it was relaxed a measurement was taken. In order to measure individuals accurately,

they were not measured until the newt remained still and relaxed (as in Figure 9, above).

3.2.2 Belly pattern analysis

Each belly pattern photograph was cropped to include the underside of the head and belly of the newt. Using the Adobe graphic design package InDesign, these were copied and pasted so that up to ten images could be printed on one side of A4 paper. It was essential that each photograph could be accurately linked to a particular individual that was captured or recaptured. Therefore each photograph was annotated using the date of the capture visit, a number to denote the order in which the newts were captured and a suffix to confirm the sex of the individual, for example 2-3.5.13_6m, denoted that it was the 6th individual, a male, captured between May 2nd and 3rd May 2013. Recaptured individuals were also named to make it easy to track newts which had been seen several times. This proved to be a thorough system of identifying individuals as there was no confusion arising from it (Figure 10).

3-4.5.13_3m 1-2.6.13_10m 5-6.7.13_2m

Figure 10: the same individual (named Arthur Lane) captured on three occasions at Lane Head Farm

Difficulties were encountered in naming individuals consistently between years. This was possible at the Garden pond since the number of captures each year were small. At Gorse Hill, for example, there were significantly more captures (741 adults in 2016). There was not sufficient time to compare these to captures from previous years, so different individuals could have been given the same name in different years. It has therefore only been possible to estimate survival at the garden pond. This data was analysed using the Programme MARK (White & Burnham, 1999) to give measures of survivability and detectability. Annual survival is the estimated proportion of animals alive in a given year that is still alive one year later (Olesiuk et al., 1990). Detectability is the chance of seeing a particular individual during monitoring (MacKenzie, 2005). It also gave population estimates which could be compared with those calculated with using the Begon Weighted Mean. Images of newt belly patterns from different dates were compared visually to find evidence of recaptures. This provided the basis of a population estimate at each pond. Population estimates were obtained using Begon's weighted mean (Begon, 1979): This method was chosen as it has been widely used by other amphibian population studies (for example Arntzen, 1993 and Jiang, 2015).

$$N = \frac{(\Sigma M_i n_i)}{[(\Sigma m_i) + 1]}$$

Standard Error (SE) was calculated using the formula:

$$SE = N\sqrt{\frac{1}{(\Sigma m_i + 1)} + \frac{2}{(\Sigma m_i + 1)^2} + \frac{6}{(\Sigma m_i + 1)^3}}$$

N = estimated population size

n_i = number of individuals captured

m_i = number of marked individuals

M_i = the number of marked individuals

The Begon Weighted Mean uses data collected over a number of successive trapping sessions and makes the assumption that the population is closed and has neither births nor deaths for the duration of the study. At each pond the objective

was to conduct a capture visit every two weeks, but in reality the survey interval was variable. In some cases it was longer (a maximum of 3 weeks at Gorse Hill and Acorn Bank in 2015) and others it was shorter (the most frequent visits were made to the garden pond, which was visited weekly in 2015 and 2016. All capture dates are shown in Appendix 5. Like most mark-recapture models, the Begon Weighted Mean assumes that all individuals are equally likely to be caught. For the purposes of this study it has been assumed that all *T. cristatus* individuals captured in one pond are part of a discreet population. This assumption has been necessary to calculate a population estimate for each pond but it is recognised that a small number of individuals at some sites are likely to disperse between ponds, particularly between years. However, dispersal between years is unlikely to affect the population estimates as they were calculated on an annual basis.

Given the capture techniques used in this study (i.e. submerged traps used throughout the breeding season) juveniles have a higher likelihood of being captured towards the end of the trapping period and sub-adults are unlikely to be captured as most do not return to the pond until they are sexually mature. Both juveniles and sub-adults have therefore been excluded from the population study. Population estimates were calculated for the whole population and then for males and females separately.

3.3 Results

Some ponds surveyed in 2013 (for example the Dead Sheep pond at Marlings and the Car Park pond at Moss Shaw Farm) provided no captures. At Seddon Fold Farm *T. cristatus* was present in only one out of eight ponds, whereas for example Marlings, it was present in six out of eight ponds. Other ponds provided very few captures (for example the Horsetail pond at Marlings where four individuals were captured and one recaptured over four capture visits). None of these ponds were re-visited in 2014 as they were thought unlikely to yield much useful data and they have been excluded from the table of results. A total of 5020 newts (including juveniles) were photographed and weighed between 2012 and 2016. Those newts captured between 2014 and 2016 (4407 individuals) were also measured. A summary of

captures is shown in Tables 7. A further 229 newts were captured from HB, R and RC, but population estimates were not calculated for these populations hence they are not included in Table 7 or 8.

The number of captures in 2013 and 2014 (542 and 660 respectively) was much lower than in 2015 and 2016 (1910 and 1623 respectively). To some extent the inclusion of Acorn Bank in 2015 increased the number of captures for that year, but the main reason was an improvement in capture technique involving night torching and emptying traps at night. The increased number of captures increased the accuracy of the population estimates (for example reducing the SE at Wittlestone Head from +/- 159.41 in 2014 to +/- 20.47 in 2016). However, the increased number of captures required significantly more time for the belly pattern photographs to be compared. Due to a lack of time it has been impossible to compare the belly patterns of the 741 newts captured at Gorse Hill in 2016.

Despite six trapping visits to Moss Shaw Farm in 2013 and the capture of 42 individuals from the same pond there were no recaptures. It was therefore not possible to calculate a population estimate in that year. In the case of Marl and LHs, no males were recaptured in 2014, and a population estimate for females and males and females combined has been produced. Population estimates for Lane Head East and Marlings Hedge pond were considerably higher in 2014 than 2013. The 2013 estimates are likely to be significant underestimates of population size whereas those for 2014 are more likely to provide a true representation. Few juveniles were captured during the study. The largest number (35) was recorded at SF in 2015, followed by 14 juveniles at GH in 2015, and 14 at SF in 2013.

3.3.1 Population size estimates at farmed and non-farmed sites

Population estimates for each study site are shown in Table 8 and Figure 11. A total of 33 population estimates were produced for 11 ponds. It was not possible to produce a population estimate for Moss Shaw Farm in 2013 due to a lack of recaptures. In 2014 there were no male recaptures at LHs and Marl, and no male

population estimates could be calculated. In 2016 it was not possible to ascertain the identity of recaptures at Gorse Hill due to the large number of captures.

Table 7: Total number of *T. cristatus* captures in 2013. Numbers in brackets are the number of different individuals identified.

Year	Site name	Total adult captures	Males	Females	Juveniles	Number of visits
2012 Totals	Bgp	25	11	14	0	8
2013	Bgp	78 (30)	25 (10)	53 (20)	0	10
2013	GH	98 (92)	73 (71)	24 (21)	1	5
2013	LH	115 (68)	26 (18)	88 (50)	1	9
2013	LHs	14 (10)	10 (7)	4 (3)	0	3
2013	Marl	78 (73)	54 (50)	24 (23)	0	5
2013	Mhp	31 (21)	15 (13)	12 (8)	4	6
2013	MS	42 (46)	21	15	6	6
2013	SF	86 (41)	25 (18)	44 (23)	17	8
2013 Totals		542	249	264	29	52
2014	Bgp	50 (23)	14 (7)	36 (16)	0	9
2014	GH	220 (192)	160 (139)	60 (53)	0	5
2014	LH	95 (56)	18 (13)	73 (43)	4	5
2014	LHs	14 (13)	9 (9)	5 (4)	0	3
2014	Marl	67 (59)	23 (23)	44 (36)	0	4
2014	Mhp	29 (19)	14 (12)	15 (7)	0	3
2014	MS	60 (44)	40 (26)	20 (18)	0	5
2014	SF	41 (34)	22 (17)	19 (17)	0	4
2014	WH	84 (75)	45 (39)	39 (36)	0	4
2014 Totals		660	345	311	4	42
2015	AB	585 (234)	341 (130)	244 (104)	0	5
2015	Bgp	49 (15)	31 (4)	18 (11)	5	18
2015	GH	610 (311)	440 (237)	170 (74)	14	8
2015	LH	81 (29)	34 (14)	47 (15)	2	8
2015	LHn	13 (10)	7 (5)	6 (5)	0	3
2015	Marl	96 (80)	58 (47)	38 (33)	0	7
2015	Mhp	52 (41)	17 (14)	35 (27)	0	7
2015	MS	75 (64)	40 (35)	35 (29)	0	6
2015	SF	103 (69)	59 (25)	44 (44)	35	9
2015	WH	245 (153)	150 (93)	95 (60)	7	10
2015 Totals		1909	1177	732	63	81
2016	Bgp	118 (19)	50 (7)	71 (12)	7	16
2016	GH	692	426	266	13	9
2016	LH	132 (45)	26 (15)	106 (30)	6	6
2016	LHn	20 (14)	8 (5)	12 (9)	0	5
2016	SF	114 (18)	84 (15)	30 (3)	8	8
2016	WH	404 (200)	211 (112)	193 (88)	8	8
2016 Totals		1532	168	412	29	47

Table 8: Population estimates from 2012 to 2016. (Min-Max = minimum and maximum number of captures per visit.)

Study site	Estimated population size	Min-Max	Estimated male population	Min-Max	Estimated female population	Min-Max
AB 2015	282.1+/-15.1	64-126	140.3 +/- 9.7	52-98	145.6 +/- 12.4	12-76
Bgp 2012	28.5 +/- 4.2	2-15	12.3 +/- 2.3	2-8	17.4 +/- 4.4	1-8
Bgp 2013	42.0 +/- 5.9	1-24	13.5 +/- 3.5	1-8	27.8 +/- 4.8	0-16
Bgp 2014	31.9 +/- 6.4	1-12	8.8 +/- 3.9	0-4	22.2 +/- 5.3	0-9
Bgp 2015	15.0 +/- 2.2	1-10	4.0 +/- 1.0	0-4	11.0 +/- 2.1	0-7
Bgp 2016	19.0 +/- 1.9	3-13	7.0 +/- 1.1	1-6	12.0 +/- 1.56	1-9
GH 2013	665.5 +/- 381.1	3-46	695.3 +/-919.8	1-38	67.3 +/-59.3	1-8
GH 2014	753.0 +/- 29.2	11-74	492.4 +/- 93.1	7-58	263.4 +/- 131.7	4-16
GH 2015	159.0+/- 9.2	4-124	148.0 +/- 10.4	3-89	35.3 +/- 5.0	1-35
LH 2013	110.1 +/-16.6	2-27	36.6 +/- 15.0	1-9	75.5 +/- 12.6	1-22
LH 2014	78.9 +/- 13.4	11-36	18.8 +/- 10.8	1-9	57.2 +/- 10.8	10-27
LH 2015	39.3 +/- 5.6	1-23	19.4 +/- 4.6	1-10	19.7 +/- 3.6	2-13
LH 2016	51.1 +/-5.6	13-30	22.5 +/- 7.5	1-5	33.5 +/-3.9	11-23
LHe 2013	12.4 +/- 8.5	3-7	7.8 +/- 6.8	2-5	2.5 +/- 7.5	1-2
LHe 2014	52.5 +/- 157.5	2-7	No recaptures	0-6	6.0 +/- 18.0	1-4
LHn 2015	24.0 +/- 21.2	2-7	8.0 +/-10.6	1-3	24.0 +/- 21.2	1-4
LHn 2016	19.1 +/- 9.6	5-9	5.8 +/- 5.1	1-4	11.5 +/- 10.1	2-6
Marl 2013	331.4 +/- 65.7	2-42	182.4 +/- 124.9	3-34	151.5 +/- 454.5	0-10
Marl 2014	195.7 +/- 74.3	8-27	No recaptures	2-11	84.6 +/- 34.7	7-19
Marl 2015	261.7 +/- 70.0	2-34	136.7 +/- 45.7	1-24	118.7 +/- 68.0	1-10
Mhp 2013	32.9 +/- 12.5	1-8	39.7 +/- 52.5	0-5	11.8 +/- 8.1	0-3
Mhp2014	129.0+/- 170.7	8-12	42.5 +/- 127.5	1-7	59.5 +/- 178.5	3-7
Mhp 2015	127.9+/- 45.4	1-18	53.3 +/- 70.6	0-10	69.7 +/- 28.6	1-12
MS 2013	No recaptures	1-16	No recaptures	1-13	No recaptures	1-3
MS 2014	225.9 +/- 92.6	1-25	164.6 +/- 112.7	1-15	80.0 +/- 105.8	1-10
MS 2015	245.3 +/- 82.0	3-20	146.0 +/- 83.6	1-14	90.4 +/- 45.2	2-11
SF 2013	70.0 +/- 13.8	1-20	39.8 +/- 17.8	0-9	33.0 +/- 7.6	1-11
SF 2014	123.6 +/- 55.5	3-16	47.8 +/- 27.4	1-10	71.7 +/- 94.8	2-6
SF 2015	100.0 +/- 16.2	4-23	30.3 +/-5.8	2-16	242.8 +/- 166.2	1-9
SF 2016	85.8 +/- 12.3	4-22	50.7 +/- 8.0	3-16	39.1 +/-14.8	1-6
WH 2014	417.2 +/- 159.4	11-30	174.4 +/- 87.2	3-18	224.3 +/- 197.8	8-12
WH 2015	193.0+/- 47.0	10-43	109.9 +/- 36.8	3-30	132.0 +/- 75.6	2-15
WH 2016	290.2 +/- 20.5	6-73	173.1 +/- 17.5	3-43	116.2 +/- 11.5	3-32

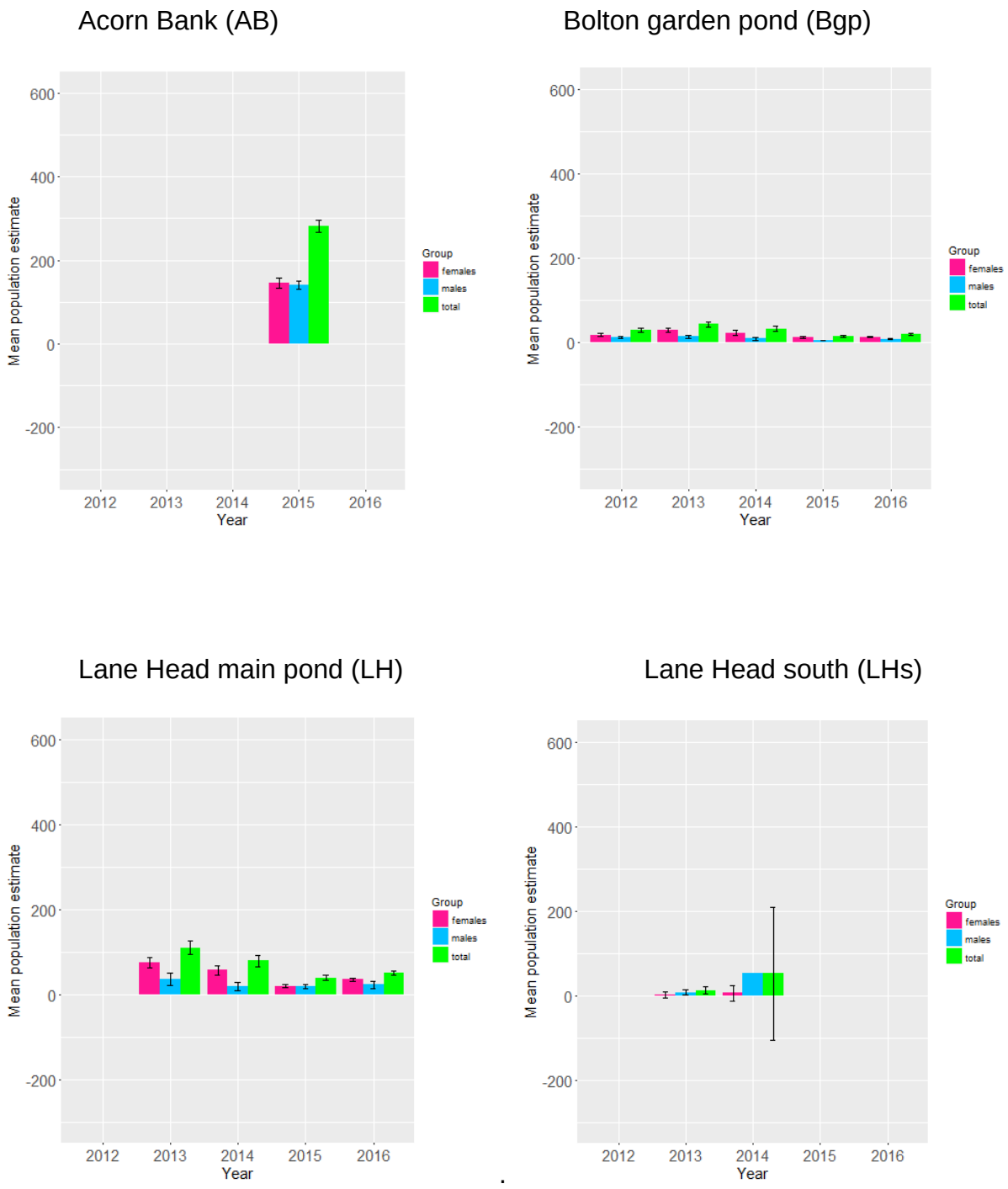
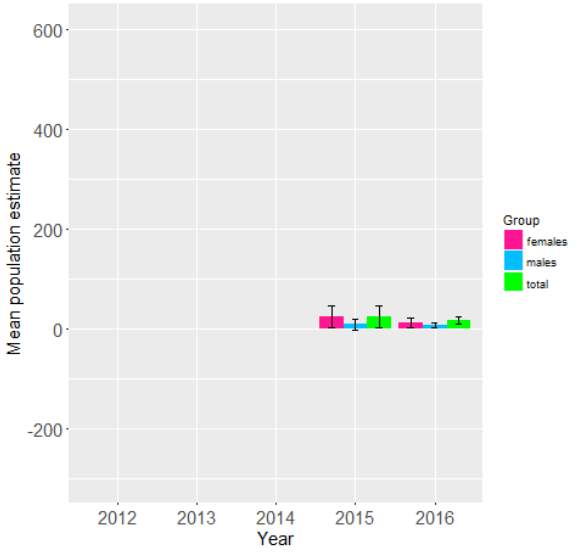
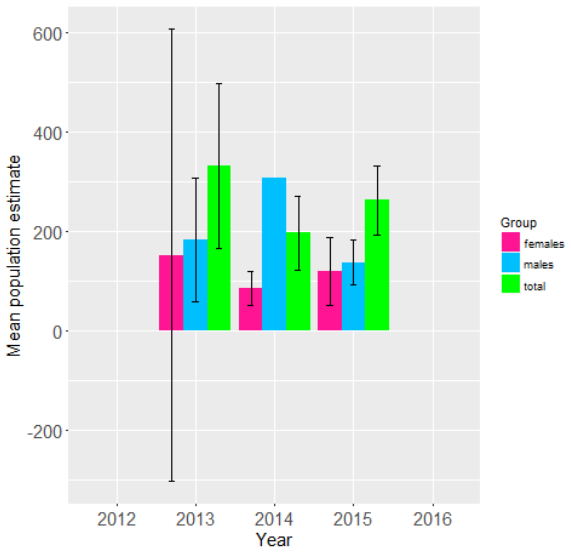


Figure 11: Population estimates showing SE bars. (No males were captured at Lane Head south in 2014.)

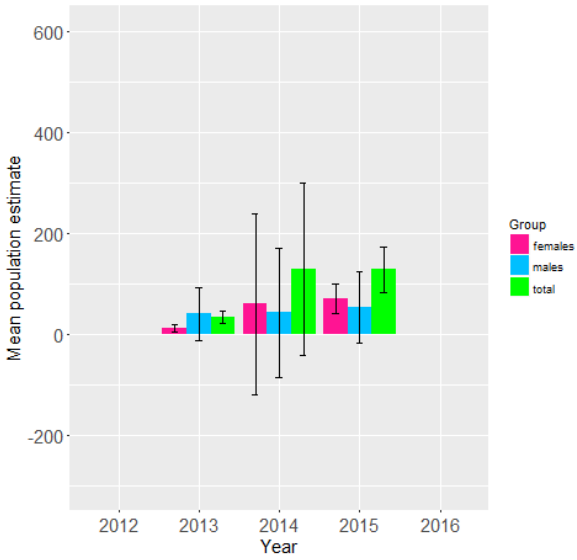
Lane Head neighbour's pond LHn)



Marlings main pond (Marl)



Marlings hedge pond (Mhp)



Moss Shaw main pond (MS)

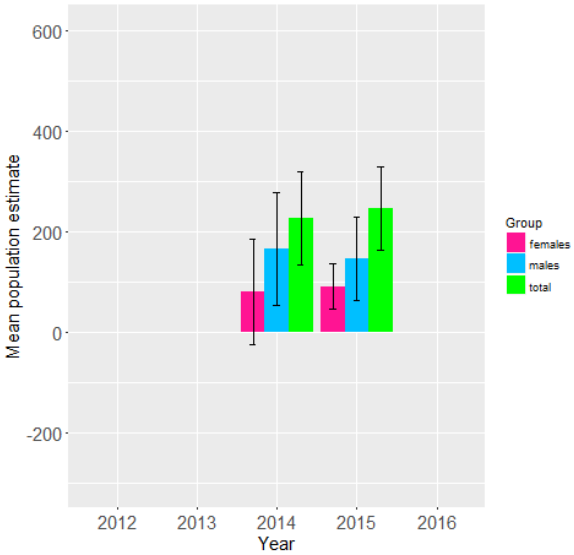
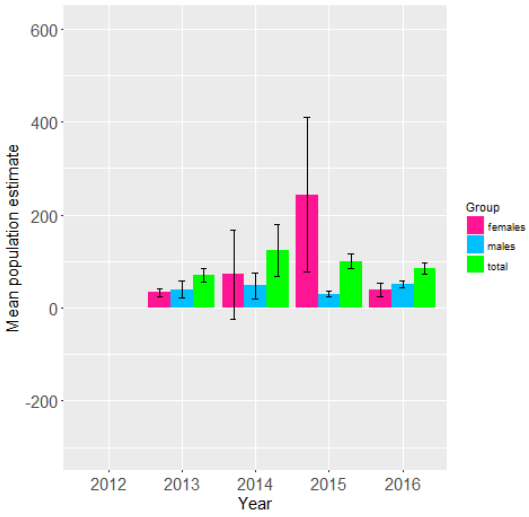
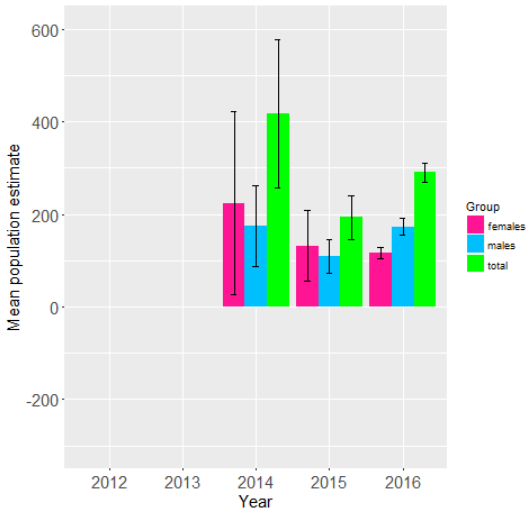


Figure 11 (continued): Population estimates showing SE bars. (No males were captured at Marlings in 2014.)

Seddon Fold (SF)



Wittlestone Head (WH)



Gorse Hill (GH)

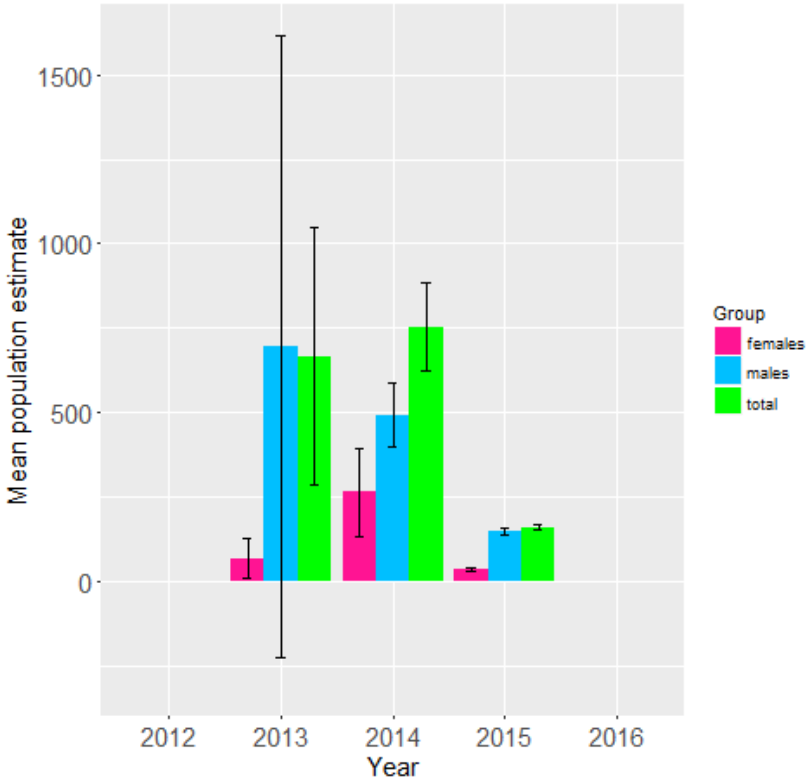


Figure 11 (continued): Population estimates showing SE bars. Note the change in scale for GH due to the large number of captures at that pond

3.3.2 The extent of population size fluctuations between years

Population size estimates varied between years. The population at Lane Head Farm declined steadily from 110 +/-16.6 in 2013 to 39 +/-5.6 in 2015, followed by an increase to 51.1 +/- 5.6 in 2016. The garden pond estimate increased between 2012 and 2013 before 3 consecutive years of decline, from 42 +/- 5.9 to 15 +/- 2.2, followed by a slight increase in 2016. Estimates at other sites showed much greater fluctuations between years. The population at Gorse Hill in 2014 of 752 +/- 29.2 fell the following year to 159 +/- 9.23. At Wittlestone Head the fluctuations were between 417 +/- 159.4 in 2014, 193 +/- 47.0 in 2015 and 290 +/- 20.47 in 2016. The population at Marlings Hedge Pond rose from 32.9 +/- 12.5 in 2013 to 127.9 +/- 45.4 in 2015.

3.3.3 Survival and detectability: a detailed study at the garden pond

The isolated garden pond provided a valuable study site as it allowed a small population to be studied in detail. Up to 40 fish nets and up to seven Dewsbury traps were used in addition to netting by torchlight. The pond was visited up to a maximum of 16 times in 2016. This gave some highly accurate population estimates and an insight into the amount of time individuals spent in the pond. During five years of data collection, a total of 55 different individuals were captured consisting of 19 males and 36 females. Details of all captures from 2012 - 2016 are shown in Appendix 6. Between 2012 and 2016, the number of new individuals captured on the final survey visit was 2, 0, 0, 0 and 0 respectively. Given the effectiveness of the capture effort it is unlikely that many newts visiting the pond evaded capture. In 2015 and 2016, the population estimates are almost the same as the total number of different individuals captured. This again demonstrates the effectiveness of the capture effort and suggests that, in 2015 and 2016, there was a high chance that all the individuals visiting the pond that year had been captured at least once. Only three individuals remained unrecorded at given years during the five year period (Sherlock in 2012, Abigail in 2013 and Debs 2016 in 2016). There was a large difference between the number of times individuals were seen in the pond. For example each of the 19 individuals captured in 2016 were seen a mean number of 6.37 times. The most frequently captured were Richard (12 times) followed by

Sharon and Phil (9 times). Least often seen were some of the new recruits to the population: Nicola2016, Aisling2016 and Debs2016 were seen on 3, 2 and 1 occasion only. This could reflect the different amounts of time individuals spent in the pond. Unrecorded individuals most likely occurred at the start of the study in 2012, when SE of population estimates was higher (4.21 – 6.38).

3.3.4 Survival at the garden pond

Using the Programme MARK, the best fitting model shows constant survival and detectability between years and sexes. Analysis revealed an annual survival of 0.64 and detectability of 0.91. The population estimates calculated with MARK and Begon's Weighted Mean were similar (Figure 12) with the main difference that MARK showed less variability between years.

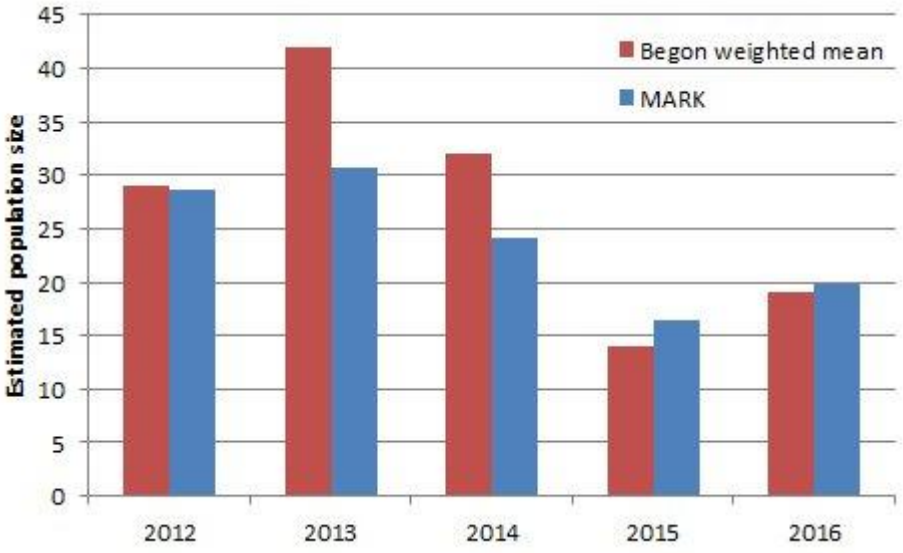


Figure 12: A comparison of population estimates using MARK and Begon Weighted Mean

Only one individual (Sharon) was captured in each of the five years of this study, and 8 individuals were captured in four years of the study. Of the 55 individuals identified, only three newts captured in different years did not return to the pond in consecutive years (Emma, Helen and Stan). It was assumed that these newts were absent from the pond in those intervening years. This suggests that the vast majority of

individuals returned to the pond each year and that if they were not recaptured following their year of first capture had been lost from the population.
Almost half of the individuals (43.6%) were captured in one year only. Seven of these were captured in the final year of the study and, due to their size and lack of previous captures, can be interpreted as young adults returning to the pond for the first time. Given that 19 newts were captured in 2016, this represents a marked recruitment and reverses the sharp decline in the population between 2014 and 2015 (estimated as a fall from 31 to 14 individuals). Some of the remaining 17 newts captured from 2012-2015 but never seen again may also have been new recruits that did not survive more than a year. The high proportion of individuals captured in one year only could indicate a relatively high mortality rate. This is reflected by the MARK data which shows annual survival of 0.64. During 2012 and 2014 the population estimates for the garden pond were very similar: 28.5 (+/- 4.2) and 31.9 (+/- 6.4). However, only six individuals from 2012 were recaptured in 2014. Five of these had also been recaptured in 2013, again inferring that the remaining newts represented a high level of recruitment.

3.3.5 Detectability at the garden pond

Capture effort during each survey visit between 2012 and 2016 was similar and very intensive. The number of capture visits increased from 8 in 2012 ,to 9 in 2013 and 2014, then to 18 and 16 visits in 2015 and 2016 respectively. Between 2012 and 2014, no new individuals were captured after the 8th visit and in 2015 only 1 was captured after the 7th. In 2016, 2 individuals were captured after the 8th visit, in June and July. Both were sub adults returning to the pond for the first time. Analysis by MARK gave a detectability value of 0.91.

3.3.6 Operational sex ratios

At three populations (the garden pond, Gorse Hill and Lane Head Farm) there was a consistent sex bias across the years. At the garden pond in 2012 the numbers were similar, with 12 males and 14 females captured, but from 2013 onwards the number of females captured was more than double the number of males. The male and

female population estimates followed the same pattern (Table 7). At Lane Head Farm population estimates for females were also markedly higher than those for males in three out of four years, but in the case of Gorse Hill the male population estimate outnumbered the female over three consecutive years.

3.4 Discussion

3.4.1 Methodological considerations

During fieldwork it was clear that a number of factors were restricting the number of capture visits that could be undertaken and hence the number of individuals that could be recorded. The need to sterilise equipment between visits to different sites was a major constraint. This procedure took a significant amount of time (2-3 hrs), and prevented one site being visited immediately after another. In 2014, the lowest number of survey visits was conducted largely due to the additional time required to toe clip individuals. Toe clipping, weighing and measuring newts often took most of the day, leaving insufficient time to sterilise all the traps, travel to another site and deploy the traps before nightfall. In such cases only one capture visit could be made over a two day period. In 2014, an improvement to routine of morning trap removal at LHe and Mhp was made which reduced the chance of individuals escaping. Consequently more individuals were captured and population estimates were considerably higher in 2014 than 2013. A further improvement to the trapping methodology at all ponds was made in 2015 with netting and the checking of traps by torchlight. This helped to markedly increase the number of captures, for example at GH rising from 98 and 220 captures, in 2013 and 2014 respectively, to 610 and 692 captures in 2015 and 2016.

3.4.2 Population estimates at the farmed and non farmed sites

Like most C-M-R models, the Begon Weighted Mean assumes that all individuals are equally likely to be caught and that populations were closed between capture visits. However, in reality it is inevitable that neither assumption is completely true. Some

individuals are more likely to be captured than others and some are likely to arrive in the pond after the first visit and/or leave before the final visit.
When comparing population estimates between ponds, the large variations in SE need to be borne in mind. The lowest SE, due to the large number of capture visits and the small population, was for the garden pond in 2015 and 2016. In contrast, the largest standard error was for Gorse Hill in 2013, the largest population with a relatively small number of recaptures. The actual size of the SE can be expected to be proportionally larger for large populations. SE for the Bgp in 2015 was 10.05% of the population estimate whereas for GH it was 57.27%. Variation in SE also needs to be considered when comparing population estimates between years. For example at Wittlestone Head, the estimate varied between 417.2 +/- 159.41 in 2013, 193 +/- 46.96 in 2014 and 290.15 +/- 20.47 in 2015. The population estimate ranges for these years, including SE's, are above/below each other which means that a clear increase/decrease in the estimated population can be assumed.

Capture efficiency is also variable between ponds. Although the capture methodology was consistent, the timing of visits was a crucial factor. In amphibians, reproduction is strongly influenced by environmental factors, and differing ecological circumstances may give rise to variation in the duration of the breeding season (Tejedo 1992). Ideally capture visits would have been made at the optimum time to catch the maximum number of individuals, but due to resource issues this was often not possible. The effect of environmental conditions on captures is shown by the date of peak captures at Lane Head Farm in 2013 and 2014. In 2013 a peak capture of 22 newts was achieved on May 13th, whereas in 2014 36 individuals were captured a month earlier on April 13th, most likely due to a warmer spring in 2014. At Seddon Fold Farm, peak counts in 2013 and 2014 were similar (16 in 2013 and 20 in 2014) but capturing remained effective until July 18th 2013. In contrast, in 2014 the capture visits ended on June 1st when no individuals were found, resulting in a shorter trapping period compared to 2013 and limiting the number of capture visits made to each pond. At GH the population estimate in 2015 was 159 +/- 9.23, lower than the previous years (665.5 +/-381.14 in 2013 and 752.97 +/- 129.22 in 2014). While it seems unlikely that the population could fall so dramatically, the low SE in 2015 indicates that this estimate is accurate and robust. The reduction may have

been related to the timing of visits, as none could be made during a period of hot sunny weather in early May. An alternative explanation is that a large proportion of individuals did not return to the pond to breed in 2015. Support for this is given by the population estimates at Bgp, LH and WH which also fell markedly in 2015. However, estimates for the other populations did not follow this pattern, so the impact of any climatic factors does not appear clear-cut. Characteristics of the pond edges were a further complicating factor. The most difficult pond to survey was Marl due to over half the pond perimeter being inaccessible due to deep water and floating mats of vegetation. Dispersal of newts between adjacent ponds may have affected some of the population estimates but this is likely to have been a minor impact since the number of newts moving between ponds is likely to be small at given geographic distances (e.g. Jehle et al., 2005). Any such error would not affect estimates at Acorn Bank, the Garden pond and Seddon Fold as they are isolated ponds. It is also possible that not all adults returned to the pond every year.

Confirming which ponds supported the biggest populations, and therefore where survey effort should be focussed at given sites, was not straightforward. In the case of Marlings, all six occupied ponds were surveyed using comparable levels of effort, whereas the largest population was only recorded mid-way through the 2013 season. At Gorse Hill, a previous survey had confirmed that *T. cristatus* was present in all three ponds, but despite five survey visits the species could only be found in two of them. A large amount of survey effort in 2013 therefore provided data which could not subsequently be used. A small number of occupied ponds were extremely difficult to survey and after initial visits were omitted.

The population at GH was by far the largest, followed by WH, Marl and MS. The large populations at GH and WH can be explained by the proximity of excellent terrestrial habitat in close proximity to the ponds. GH was surrounded by dense vegetation and woodland. WH, although surrounded on three sides by close cropped sheep pasture, was close to an old railway cutting dominated by unmanaged scrub and woodland. The population size estimates show that the population at GH does not appear to have been constrained by its isolation within an intensively managed arable landscape.

Populations at Marl and MS were similar in size, even though the terrestrial habitat and pond characteristics were different. The pond at Marl was surrounded by 3-4m dense vegetation with hedges and a small woodland 150 m away, whereas the pond at MS was surrounded on three sides by close cropped grass and a wet field dominated by *Juncus effusus*. The pond at Marl was deep and well vegetated at the margins, whereas the pond at MS was shallow with very limited marginal vegetation. Both ponds were in a pond rich landscape. It is likely that drawing simple conclusions about population size from habitat quality is not possible. At LH, also a pond rich landscape with good terrestrial habitat consisting of low intensity pasture, traditional hedgerows still in place and 4-5m of dense vegetation surrounding the pond, the PSYM score was higher than that for Marl or MS. However, the main population was relatively small and declined between 2013 to 2016 from 110 +/- 16.6, to 50.34 +/- 5.49. It is possible that the creation of two new ponds and the management of another pond (LHn) in 2013 within 200 m of the main pond encouraged adults to disperse away from the main pond. Both new ponds and LHn were visited during 2014 and 2015, although capture effort was much lower than at the main pond due to limitations of time. One of the new ponds showed no evidence of *T. cristatus* but the other pond contained a small number of eggs (egg searches in 2016 failed to find eggs in either pond). One large adult female was captured from this pond in 2014, which was not seen anywhere else throughout this study. A maximum of 9 individuals were captured from LHn, including an adult male which had previously been seen in the main pond. LHn had been occupied by *T. cristatus* prior to the management work, so it is likely that most of the individuals captured here were present in this pond before and after management. Together these finding indicate that (i) sexually mature adults can colonise new ponds and breed successfully within one year, (ii) only small numbers of individuals are likely to be involved in colonisation, (iii) not all new ponds are colonised (the uncolonised pond was larger and closer to the main pond than the one in which the eggs and adult female were found), and (iv) a small number of adults were confirmed as dispersing between the main pond and a pond already supporting *T. cristatus*. Migration of some individuals to the new ponds is likely to account for some of the population decline, although this does not seem to account for the extent of the estimated population decrease between 2013 and 2016. During survey visits it was noted that

Notonectidae were common and that *Dytiscus marginalis* larvae were also present. These are voracious predators and may have had a significant impact on *T. cristatus* larvae.

The population size at AB was remarkably high, given that it was dependent upon a small ornamental pond no more than 4m in diameter. It was the site with the fourth largest population estimate (282.08) with one of the smallest SEs (+/-15.09), achieved due to ease of capture of newts from the ornamental pond. Individuals were easy to net by torch and almost the entire pond was within reach. A maximum of 126 individuals were captured in one night. Only one survey visit at Gorse Hill in 2016 resulted in a higher number of captures (165). Given the diameter of this pond the size of this population relative to others in this study is difficult to explain. It seems that good terrestrial habitat quality (proximity of old stone features, a garden and woodland) is likely to be responsible for the population size.

Most juveniles (35) were captured from SF in 2015, whereas no juveniles were captured at five out of 10 sites. Over the entire study a total of 108 juveniles were captured, 51 of which were from SF. The small number of juvenile captures has been reflected in other research. For example in a detailed study by Arntzen (2000) only 38 juveniles were captured compared with a total of 485 adults. The earliest date of a juvenile capture throughout this study was from SF on April 13th 2015. The large number of juveniles at SF may be related to the pond being well vegetated, as in mid-summer the water surface was completely covered by *Rorippa nasturtium-aquaticum*. It may be that this provided *T. cristatus* larvae with sufficient refuge from predators to increase their survival rates.

It is highly likely that, taken in isolation, populations at some of the ponds in this study could be too small to be viable in the long term. In the case of Moss Shaw Farm, for example, three out of the five occupied ponds support very small populations (Al, MSmud and MSsp) based on field observations and capture results. However, given they are part of a metapopulation, recolonisation from neighbouring ponds is possible. The small isolated population in the garden pond might be at risk from extinction as there is no opportunity for it to be recolonised. Nevertheless this

study has shown that the pond supported a viable population for over 20 years. Effective population size in amphibians, has generally been estimated as under a hundred individuals, whereas the minimum effective population size required to maintain genetic variation sufficient for demographically viable populations is thought to be between 500 and 5000 individuals (Franklin & Frankham, 1998; Lynch & Lande, 1998). Given that many European amphibian species are subject to increasing population isolation, these findings suggest that the long-term survival of many populations is in danger, more so than field ecological studies would reveal.

A detailed population study of the *Bufo calamita* (Denton, 1993) found that although the majority of males took part in mating choruses over the 2-3 months of the breeding season, almost as many (15 out of 38 in 1988 and 12 out of 27 in 1989) appeared at ponds for the first two weeks only. Therefore a large proportion of the population could have remained undetected if visits to the pond had started just 2 weeks later. A reason why some males are present for such a short period could be that breeding activity incurs substantial energy costs, so only the fittest males can sustain breeding activity for a prolonged period (Denton, 1993). Another study by Tejedo (1992) found that smaller males attended the breeding site less frequently than larger males. His hypothesis was that this was due to energy being allocated to growth in small males as opposed to sustained chorus attendance.

Whilst breeding behaviour in *Bufo calamita* may be very different from that of *T. cristatus*, this nevertheless illustrates that some amphibians participate in a long breeding season for only a short period. To some extent this behaviour is likely to be reflected in *T. cristatus*. For example Langton et al., (2001) reported that a third of the population at a well-monitored study site in England occupied the pond for less than 10 days during the breeding season. This could be one of the reasons why capture and recapture rates were not higher throughout this study, as capture visits were often two weeks apart.

3.4.3 The extent of population size fluctuations

Declining population estimates at Lane Head Farm and the garden pond are likely to indicate true changes in population size. The low SE gives a large degree of certainty over the statistical accuracy of the estimates and relatively small changes are likely to be part of cyclical population changes documented elsewhere (Jehle et al., 2011). The much larger fluctuations, for example at Gorse Hill, Wittlestone Head and Marlings Hedge pond, are unlikely to reflect true changes in population size and probably arise from other factors. In the case of Gorse Hill, there was no apparent reason for the huge decline in the estimated population between 2014 and 2015 but the low SE in 2015 of +/- 9.2 indicates that the estimate is a true reflection of the number of newts in the pond that spring. A similar phenomenon was reflected at Wittlestone Head. This estimated population fell by over 50% between 2014 and 2015 then rose by over 50% in 2016. Such large and rapid changes are very unlikely to reflect true changes in population size due to the longevity of individuals. Instead, such changes may be due to different numbers of newts available for capture, ie perhaps a large proportion of newts did not return to the pond in 2015, or maybe there was a huge population crash. In total, population estimates from 6 ponds declined between 2014 and 2015 (another increased and two remained constant over the same period). This suggests that the lower estimates in 2015 could be due to environmental factors. If the lower estimates were due to newts not returning to the pond to breed in 2015, this would contradict evidence from the garden pond which suggested that the majority of surviving individuals returned to the pond each year. A closer look at the garden pond population between 2014 and 2015 showed a fall from 31.9 +/- 6.4 in 2014 to 15 +/- 2.2 in 2015. Of 23 different individuals captured in 2014, 11 were not seen again in either 2015 or 2016 which indicates that this population had undergone a genuine fall between 2014 and 2015, Unlike Wittlestone Head it did not show a marked increase in 2016. It is possible that environmental factors affected *T. cristatus* between the breeding season on 2014 and 2015. In some populations this led to a fall in the population but in others the effect was temporary. The reasons for this require future investigation.

The increase in the population estimate for Marlings Hedge pond is most likely a result of changes in capture technique and it is very unlikely that the population increased four-fold in the space of one year. In 2013, 21 different individuals were captured but in 2015 the number increased to 41 due to improved capture efficiency. This is the most likely reason for the apparent increase in the population at this pond.

3.4.4 Survival and detectability: a detailed study at the garden pond

Data from this study shows that of the 55 different individuals identified, only one (a female) was captured in all five years and only eight were captured in four years of the study. The reasons for this can only be speculated, but perhaps those newts which returned in several consecutive years resided close to the pond during their terrestrial phase where the risk of mortality is low. Newts which migrated furthest from the pond would be at greatest risk of mortality, for example by falling into gulley pots or being killed on roads and this could be a reason why relatively few newts were seen over several consecutive years. The study shows a high level of recruitment, for example in 2016, 7 of the 19 individuals captured were new recruits. This indicates that the risk of mortality in the terrestrial habitat is a key limiting factor for population size.

Over the 5 years of the study, only 3 individuals were recorded after the 8th capture visit. This demonstrates that even with intensive capture effort and high detectability (calculated by MARK as 0.91) at least 8 capture visits over April and May are required to capture each individual at least once. All other ponds in this study, with the exception of Acorn Bank, received a much lower capture effort due to the size of the pond and difficult access. This explains why some population estimates have very high measures of SE. The highest SE in this study, of 381.1, was for Gorse Hill in 2013 which received 5 capture visits. The benefit of conducting a minimum of 8 visits depends on the research objective, which in this study was to obtain a population estimate to a high degree of statistical accuracy. At Lane Head in 2016 it was possible to obtain a population estimate with a SE of only 5.6 after just 6 visits, illustrating that 8 visits may not be required to obtain a reliable population estimate.

Additional visits, to a maximum of 18 at the garden pond, were useful to confirm that few newts had evaded capture.

In order to avoid wasted survey effort by conducting more capture visits than required to obtain a statistically accurate population estimate, the best approach would be to compare belly patterns and calculate the estimate after each visit. As soon as the SE error becomes low (which needs to be proportionally smaller for small populations), capture visits can be discontinued. In practice this is difficult due to the very limited time available between capture visits and it was not possible during this thesis. Fewer visits are likely to be needed where capture effort is high and for populations with high detectability.

3.4.5 Operational sex ratios

European urodeles of the genus *Triturus* usually have a sex ratio of 1:1 (Jehle et al., 2011; Sinsch, 2003). This ratio is reflected in some but not all of the population estimates calculated by this study (Table 7). This apparent sex imbalance could be related to capture methods biased in favour of either sex. Beebee (1990) found that male *T. cristatus* were more easily caught using bottle traps than females (a ratio of approximately 3:2). If this experience was typical, it could be expected that any sex bias of captures should be in favour of males, which was not the case in this study. A possible explanation for sex bias in capture results is that males or females were captured early during the trapping session, attracting newts of the opposite sex. However a study by Rödel et al., (2014) found that the presence of males or female *Lissotriton vulgaris* or *Ichthyosaura alpestris* in traps did not affect the number of subsequent captures. If capture methodology was indeed the reason for sex bias, it would further impact on the sex specific SE of the population size estimates. It would also be reasonable to expect that either the male or female populations would be consistently larger throughout this study. However neither of these statements is true. Such differences in sex ratios have been found in other studies (Jarvis, 2012). In 2009 he captured four times as many females as males and in the remaining seasons (except 2007 when ratios were more equal), females dominated males, possibly due to the low numbers of total captures. Also, Jarvis (2012) did not

estimate population size for males and females at each pond, so his assumption that the number of captures represents population size may well be incorrect. It seems likely that some populations do exhibit a sex bias in favour of either males or females as a characteristic of those populations. For example at Gorse Hill between 2013 and 2015, male population estimates varied between 695, 492 and 147 respectively while female estimates over the same period were 67, 263 and 35. Whether this is a temporary or long term phenomenon is beyond the scope of this study.

A factor likely to affect the extent to which the population estimate is truly representative is uncertainty over the proportion of individuals that return to the pond each year. It is possible that not all females return every year. For example, in *Bufo bufo*, if female body condition is not restored between breeding periods their next ovarian cycle is not possible. In such cases females may not be able to breed the following year (Denton & Beebee, 1993). A similar process may occur to some extent in *T. cristatus*.

Chapter Four: The age structure and body condition of *T. cristatus* populations in the farmed landscape

Summary

Age structure is an important component of the demography of a population. A number of previous studies have been conducted into the age structure of *T. cristatus* populations using skeletochronology. The present study is however the first in the UK, and probably the largest in Europe, including age estimates of 548 newts from 13 populations on 11 sites. In addition to age data, the weight and length of 4693 individuals was measured between 2014 and 2016 to serve as a measure for body condition. Ages ranged from three to 15 years, and individuals reached sexual maturity at between two and four years. Few individuals of 5 years old and below were captured and the median age was seven years, which suggests that many individuals do not return to the pond until several years after reaching sexual maturity. Individuals over 10 years old were captured on average three weeks earlier than those aged 4-5 years. Age of individuals could not be estimated from their length. Body condition of both males and females overall decreased with age.

4.1 Introduction

Determining the age structure of a population is important for assessing its conservation status, as for example a senescing population is at risk of decline in the near future (Jehle et al., 2011). When amphibians have to live under anthropogenic influences, studies on the age composition of populations are important for biomonitoring of the environment (Smirina, 1994). However, a long-standing challenge for amphibian population ecologists is the reliable estimation of age in individuals without known recapture history (Sinsch, 2015). To reduce the uncertainty of adult and juvenile survival rates, *T. cristatus* is in need of additional demographic studies, ideally combined with C-M-R methods to obtain reliable estimates of survival (Schmidt, 2003). Improved demographic data are also required to produce refined population viability models (Karlsson, 2007).

Skeletochronology is the technique of estimating the age of individuals by counting annual growth rings, or annuli, within thin bone cross sections. This approach has been used to estimate the age of amphibians, (Castanet, 2002), dinosaurs (Horner et al., 1999), birds (Bourdon et al., 2009) and mammals (Marin-Moratalla et al., 2013). In the past, long bones such as the humerus and femur were used (Dolmen, 1982; Hagstrom, 1980) but this requires unacceptably large numbers of individuals to be sacrificed. Non-destructive age determination became possible after Smirina (1972) demonstrated that phalanges obtained by toe-clipping also provide the same information. Since then, a large number of skeletochronogical studies on amphibians have been published, mainly focusing on the population ecology of temperate-zone anura and caudata (Sinsch, 2015). The technique of skeletochronology is based on lines of arrested growth (LAG), which are formed each year and correspond to the age of the individual. The cause of annual LAG formation is a genetically based circannual rhythm synchronised with seasonal cycles (Castanet et al., 1993). Therefore LAGs are also formed in tropical habitats with very little seasonality (Khonsue et al., 2000). Sometimes bone sections show broad, faint annuli (Castanet et al., 1993; Alcobendas & Castanet, 2000) within long-lasting growth periods. These lines of reduced growth (LRG) indicate slower but not arrested growth (Sinsch et al., 2007) and they can complicate age estimation.

There are two standard methods for determining the age of amphibians: skeletochronology and long term C-M-R investigations (Wagner et al., 2011). Due to the relatively high longevity of amphibians, mark-recapture data require years (if not decades) of field work. The more practical method is therefore skeletochronology, although some studies suggest that this can lead to an underestimation of age in newts and other amphibians (see Wagner et al., 2011; Sinsch, 2015). Despite these constraints this method is nevertheless the most reliable method for age determination in newts (Castanet & Smirina, 1990) and it is a standard method for numerous studies of ageing amphibians (for an early review see e.g. Halliday & Verrell, 1988). The method relies on obtaining a digit from each newt which is then cut into a thin section allowing the lines of annual growth (LAGs) to be counted.

Newts have the ability to regenerate digits within approximately one season, to the extent that effects of toe clipping can be difficult to see after a few months (Henle et al., 1997). A study on the neotropical frog *Allobates femoralis* suggested that amphibian toes re-grow more quickly in young individuals than older ones (Ursprung et al., 2011) but there have been no specific studies published on the regrowth of toes in *T. cristatus.* Toe clipping has no effect on survival and body condition of *T. cristatus* (Arntzen et al., 1999), but the ethics of removing toes for scientific research remains controversial.

Miaud et al., (1993) showed for *T. cristatus* that age structures can differ widely between adjacent populations, and that they can fluctuate over time. Other studies have shown regional variations in maturity and longevity. The juvenile stage of *T. cristatus* may last from two to five years (Smith, 1964; Dolmen, 1982; Francillon-Viellot et al., 1990). Generally *T. cristatus* from higher elevations or northern latitudes attain higher ages than those from lower elevations or from southern regions. For example in Scandinavia, newts reach maturity after 3-4 years (occasionally five) years, on average attaining 7-8 years (Hagstrom, 1977). Crested newts from France mature earlier but also grow faster and reach maturity at a larger size than their Scandinavian counterparts (Arntzen, 2000). Studies using skeletochronology have shown that *T. cristatus* males can live up to 17 years and females up to 16 years (Dolmen, 1982; Miaud et al., 1993). Estimated ages for *T. cristatus* across Europe are shown in Table 9.

Body condition indices give an indication of the relative health of an individual and may be useful in assessing the current status of a population and the quality of individuals (Janin et al., 2011). Studies examining body condition in adult amphibians are widespread (e.g. Baker, 1992; Cooke & Arnold, 2003; Kopecký et al., 2010; Lowe et al., 2006) and, due to the costs of reproduction, generally suggest that amphibians have a higher body condition at the start of the breeding season compared to the end (Arntzen et al., 1999). There are several methods of calculating BCI (see e.g. Labocha, 2014, comparing 17 different methods used for mammals).

Table 9: The age of *Triturus cristatus* as determined by skeletochronology in various populations across Europe. *n* = number of individuals studied. Min-max = recorded range of adult ages; median = median age (taken from Jehle et al., 2011).

Population origin	*n*	Min-Max	Median	Reference
Males				
Goteburg (Southwestern Sweden)	43	3-16	7	Hagstrom (1977)
Trondheim (Central Norway)	47	4-16	8	Dolmen (1982)
Koblenz (Western Germany)	91	2-11	4	Sinsch et al., (2003c)
Cologne (Western Germany)	22	2-7	4	Schlagheck (2002)
Mayenne (western France)	47	1-14	3	Francillon-Vieillot et al., (1990)
Bresse (Eastern France), year 1	52	2-17	5	Miaud et al., (1993)
Bresse (Eastern France), year 2	120	2-17	4	Miaud et al., (1993)
Females				
Goteburg (Southwestern Sweden)	43	3-13	7	Hagstrom (1977)
Koblenz (Western Germany)	107	2-9	4	Sinsch et al., (2003c)
Cologne (Western Germany)	35	2-7	5	Schlagheck (2002)
Mayenne (western France)	39	1-9	3	Francillon-Vieillot et al., (1990)
Bresse (Eastern France), year 1	36	2-16	5	Miaud et al., (1993)
Bresse (Eastern France), year 2	102	2-12	4	Miaud et al., (1993)

Simple ratios between mass and a linear measure of body size are often used (Mateo et al., 1998; Whitfield et al., 1999) but problems with ratio methods have been identified (Ranta et al., 1994; Jakob et al., 1996). Lewis (2012) used a model II linear regression of *T. cristatus* log body mass versus log length residuals as measures of body condition, to control for variation in length (Băncilă et al., 2010). Another useful method of calculating body condition has been recommended by Green (2001), plotting Log10(SVL) against Log10(Weight). This method was also used by Jarvis (2012) for the great crested newt and was applied in the present study.

4.1.1 Research objectives

This research investigated the longevity and body condition of individuals at farmed and non farmed sites as any differences could indicate advantages for *T. cristatus* of one environment over the other. This study had two objectives; (i) A comparison of

the age structure of populations at farmed and non farmed sites. (ii) A comparison of the body condition of individuals at farmed and non farmed sites. Using this data it was also possible to investigate the effect of age upon the date of capture and to compare age with body condition.

4.2 Methods

4.2.1 Methods to compare the age structure of populations at farmed and non farmed sites.

In 2014 and 2015, toe clips were taken at 12 ponds. Nine of these were ponds for which population estimates and genetic data were available (see Chapters 3 and 5). To provide a more thorough comparison of populations on farmland with those on favourably managed sites, the control sites of GH and Bgp were supplemented by toe clips taken from three additional sites, HB, R, RC and AB. Independent surveys have counted over 100 adults by torchlight at each of these sites. Therefore, they can be assumed to support large *T. cristatus* populations. In cases where it was not possible to toe clip 50 individuals (25 males and 25 females) per pond, all adults captured were toe clipped. A summary of age estimates obtained is shown in Table 9.

A Home Office licence is required for any procedure that involves more stress to an animal than the insertion of a hypodermic needle (Animals (Scientific Procedures) Act, 1986). A personal licence was obtained in October 2013 (see Appendix 7). A Home Office project licence is also required, and this must be held by a person from an institution with a Home Office Establishment Licence. As the University of Salford does not hold a project licence, it was obtained in partnership with the Institute of Zoology (project licence holder Dr. Trent Garner). A total of 548 age estimates was obtained as part of this project (Table 10).

Table 10 The number of age estimates obtained for each population

Pond Reference	Male samples	Female samples	Total samples
AB	25	28	53
Bgp	6	18	24
GH	33	19	52
HB	12	21	33
LHs	12	25	10
LH	7	3	37
Mhp	29	27	47
Marl	20	27	56
MS	23	27	50
RC	18	22	40
R	19	18	37
SF	25	25	50
WH	25	18	59
Total	**259**	**289**	**548**

For the 2014 field season, the third toe of the left and right foot was removed in 2014 and 2015, respectively. The third digit was invariably the largest, increasing the chance of accurately interpreting the age of the individual. The third, largest, segment of the toe was used to identify the lines of arrested growth (LAGs); the second digit could be used in cases where the third segment was deformed. The first segment (i.e. the tip of the toe) was removed and retained for future genetic research.

Digits were removed using a sharp scalpel and the cut was made on the joint at the base of the toe, allowing the whole toe to be taken (Figure 13). Care was taken to avoid cutting into the skin or bone above the toe. The open wound was treated by spraying with Bactine, an antiseptic spray, and the clipped newts were released back into the pond. Toe clipping was done after the newts had been weighed, measured and photographed to avoid causing undue stress to the injured animal. Each toe clip was stored in its own tube containing 70% alcohol. The scalpel was sterilized using a flame from a cigarette lighter before being used again, thus preventing contamination of toes with DNA from other individuals. A total of 548 age estimates was obtained using this method (Table 10).

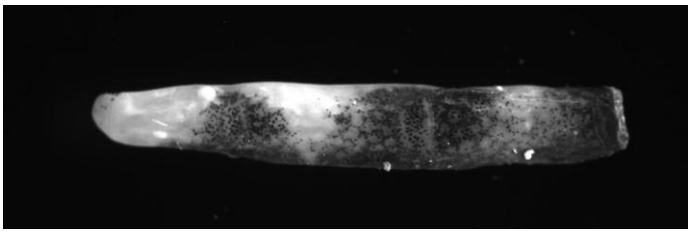


Figure 13: A toe of *T. cristatus* removed in 2014.

In 2014 it was noticed that individuals had started to re-grow toes which had been removed by the end of the season (Figure 14). The first survey visit of 2015 recaptured a newt which had been toe clipped the previous year, and the removed toe had completely re-grown (Figure 15).

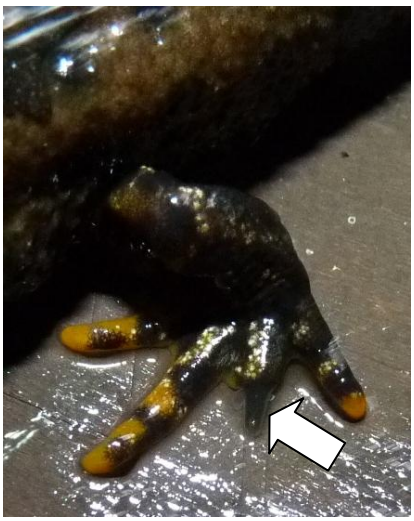


Figure 14: This individual was toe clipped on April 7th 2014 and subsequently captured another three times in that year. This photograph was taken on June 13th 2014, demonstrating that the toe was starting to re-grow within two months.

Figure 15: The individual toe clipped above was photographed again on March 5th 2015. The toe indicated is the one removed the previous year, confirming that not only can *T. cristatus* survive toe clipping but that toes can re-grow within twelve months. The same individual was captured again on twelve occasions in 2015 and a further twelve in 2016, demonstrating that toe clipping did not affect survival of the individual.

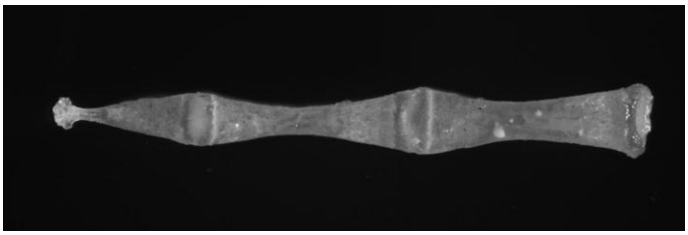


Figure 16: A toe bone of *T. cristatus* with skin removed ready for the microtome.

Preparation of the toes followed the standard laboratory procedures detailed by Sinsch (2015). Prior to the bone sections, flesh was gently scraped from the amputated toe (Figure 16), which was placed in 70% nitric acid for 75 minutes, followed by soaking them in water overnight. This made the bones softer so that they could be cut more easily into thin sections. Each toe bone was cut into thin sections of 10–16µm, using a cryostat microtome (Figures 17a-17e). The sections were stained with Ehrlich´s haematoxylin solution until growth marks became visible (e.g., Smirina, 1972; Sinsch et al., 2001).

The Cryostat microtome operates at a temperature of -20°C which prevents the ice from melting. Thin sections were stained and mounted onto slides according to the methodology described by Sinsch (2015). LAGs were counted using 200x-400x magnification. Age at sexual maturity was considered as the youngest age at which inter-LAG spaces reduced. During juvenile growth spaces between LAGs are significantly greater than during the adult stage, as juveniles invest more in growth whereas adults invest more in reproduction (Kleinenberg and Smirina, 1969). One of the stained toe sections is shown in Figure 18.

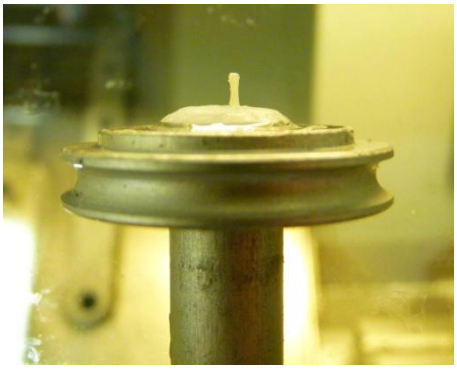


Figure 17a

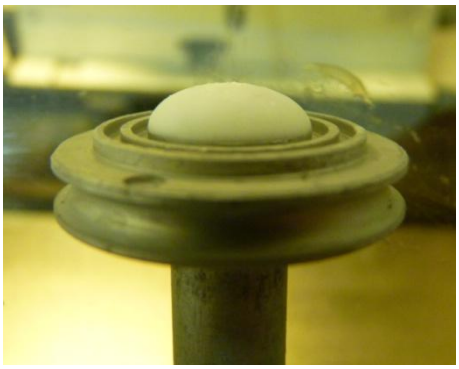


Figure 17b

Figure 17c

Figure 17d

Figure 17e

Figure 17: Methodology for cutting a toe section for *Triturus cristatus*. 17a: The toe bone held vertically by being placed on a small drop of ice. 17b: The bone encased in ice. 17c: Excess ice removed, exposing the top of the bone. 17d: The small plate clamped firmly into the cryostat microtome. 17e: Thin sections shaved from the ice by gradually moving the small plate closer to a sharp cutting edge.

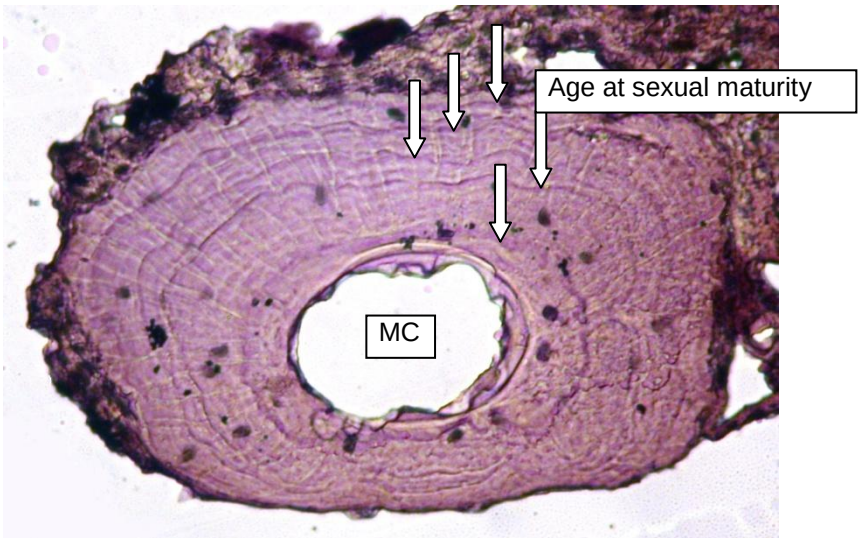


Figure 18: Stained toe sections from a male *Triturus cristatus* captured on May 2nd 2014 at Rixton Claypits. This individual was 5 years old and sexually mature at 2 years of age.LAGs are indicated with the arrows. MC = medullar cavity.

4.2.2 Methods to compare the body condition of individuals at farmed and non farmed sites.

In order to calculate BCI, each newt captured from 2014-2016 was weighed and measured. Newts were weighed and measured at the same time as they were photographed for the population estimates (see Chapter 3, Section 3.2.1 for details).

Body condition of all individuals captured in 2014-16 was calculated using the programme R. A linear model was created of Log10 (weight) plotted against Log10 (length). A locally-weighted polynomial scatter plot smoother as a line of best-fit was added and residuals between the best-fit line and the actual data points were calculated. Negative values arise from creating the line of best fit through all the data points. The line represents average body condition, therefore animals with BCI above this line have a positive BCI and those below it are represented with a negative BCI.

4.3 Results

4.3.1 Comparison of the age structure of populations at farmed and non farmed sites.

The age of 548 individuals was obtained from 13 populations on 11 sites (Table 11, Figure 19). The age at which individuals reached sexual maturity is shown in Table 11.

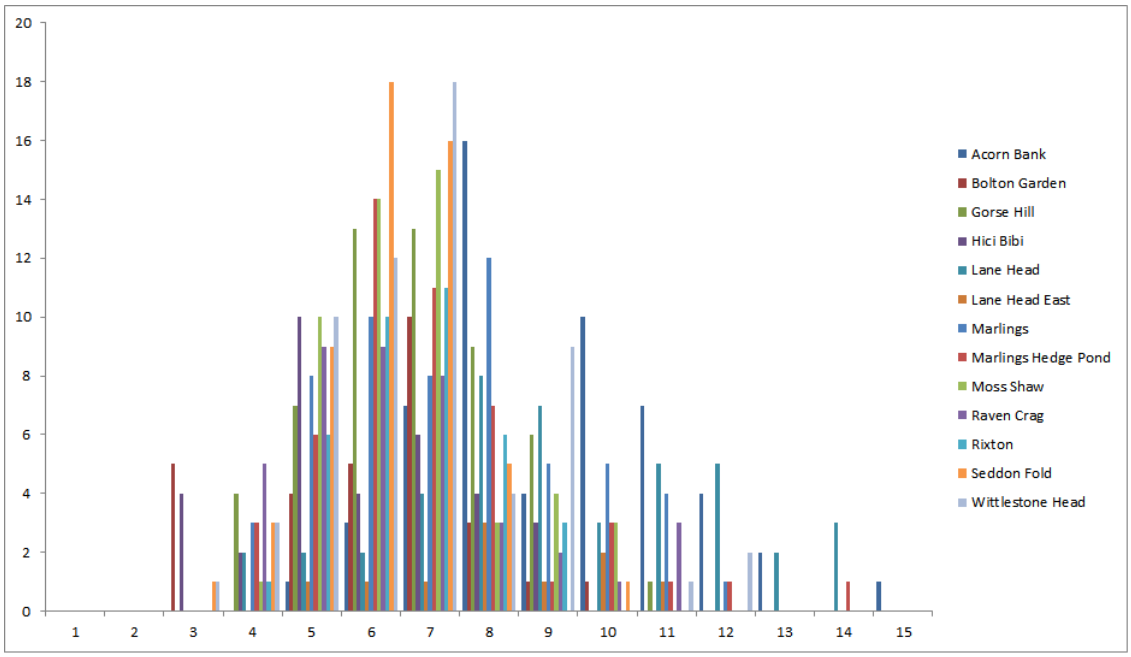


Figure 19: The estimated age of all individuals in all populations

Most individuals became sexually mature at either 2 or 3 years (Table 12). 2 matured at 1 year old, 105 at 2 years, 179 at 3 years and 14 at 4 years. Of the 20 individuals aged 12 and over, 18 were female. (Figure 20). Females lived on average longer than males (exceptions: Gorse Hill and Seddon Fold). Across all sites, mean age of females (n=286) was 7.55 years, and mean age of males (n=262) was 6.7. Males predominated up to the age of 6 years, and females predominate at age 7 and over. Of those aged 5 and under (n=116), 71 were male (61.21%) and only 7 females were aged 3 or 4 years. From age 11 (n=42), 29 individuals were female (69.01%) and all those over 13 tears were females.

Table 11. Estimated age of individuals in each population

Popn	Estimated age (years)												
	3	4	5	6	7	8	9	10	11	12	13	14	15
AB			1	2	7	16	4	10	7	4	1		1
Bgp			4	5	10	3	1	1		-			
GH		4	7	12	13	9	6		1				
HB	4	2	10	4	6	4	3						
LHs			1	1	1	3	1	2	1				
LH		2	2	2	2	7	6	3	4	4	2	3	
Mhp		3	6	13	11	7	1	3	1	1		1	
Marl		3	8	10	8	12	5	5	4	1			
MS		1	10	14	15	3	4	3					
RC		5	9	9	8	3	2	1	3				
R		1	6	10	11	6	3						
SF	1	3	9	17	15	4		1					
WH	1	2	10	12	18	4	9		1	2			
Total	**6**	**26**	**83**	**111**	**125**	**81**	**45**	**29**	**22**	**12**	**3**	**4**	**1**

Table 12. Estimated age at sexual maturity in each population

Popn	Estimated age (years)			
	1	2	3	4
AB		12	23	3
Bgp		5	13	1
GH		11	22	1
HB	1	5	9	
LHs				
LH		8	20	5
Mhp		7	4	
Marl		4	17	1
MS		2	8	
RC		18	5	1
R		6	20	1
SF		11	17	1
WH	1	16	21	
Total	**2**	**105**	**179**	**14**

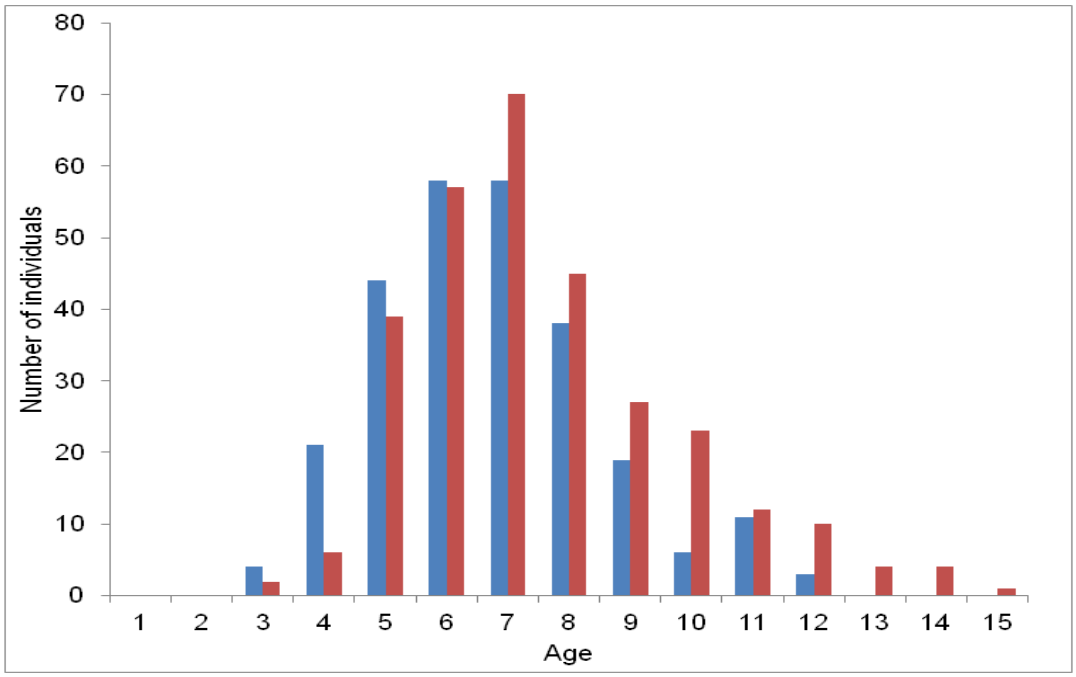


Figure 20 The estimated age of all males and all females in all populations (males in blue, females in red).

The age structure of each population is shown in Figure 21, which shows considerable variation between sites. Acorn Bank (n=53) included a high proportion of older individuals. In this population, the median age for both males and females was 8 years old, 58.4% were aged 11 or over and only 13.21% of individuals were estimated as seven years of age or younger. At Gorse Hill (n=52), the median age for males was 6 and for females was 7. Only one individual (1.92%) was 11 years old, and 69.2% were aged 7 or younger. The smallest sample size was for Lane Head east (n=10), but again longevity was higher for females than for males. There was a high degree of variability between population age structures. No distinction could be drawn between those on farmed and non farmed sites. There was no significant relationship between population size and age structure for males (p-value = 0.67) or for females (p-value = 0.82).

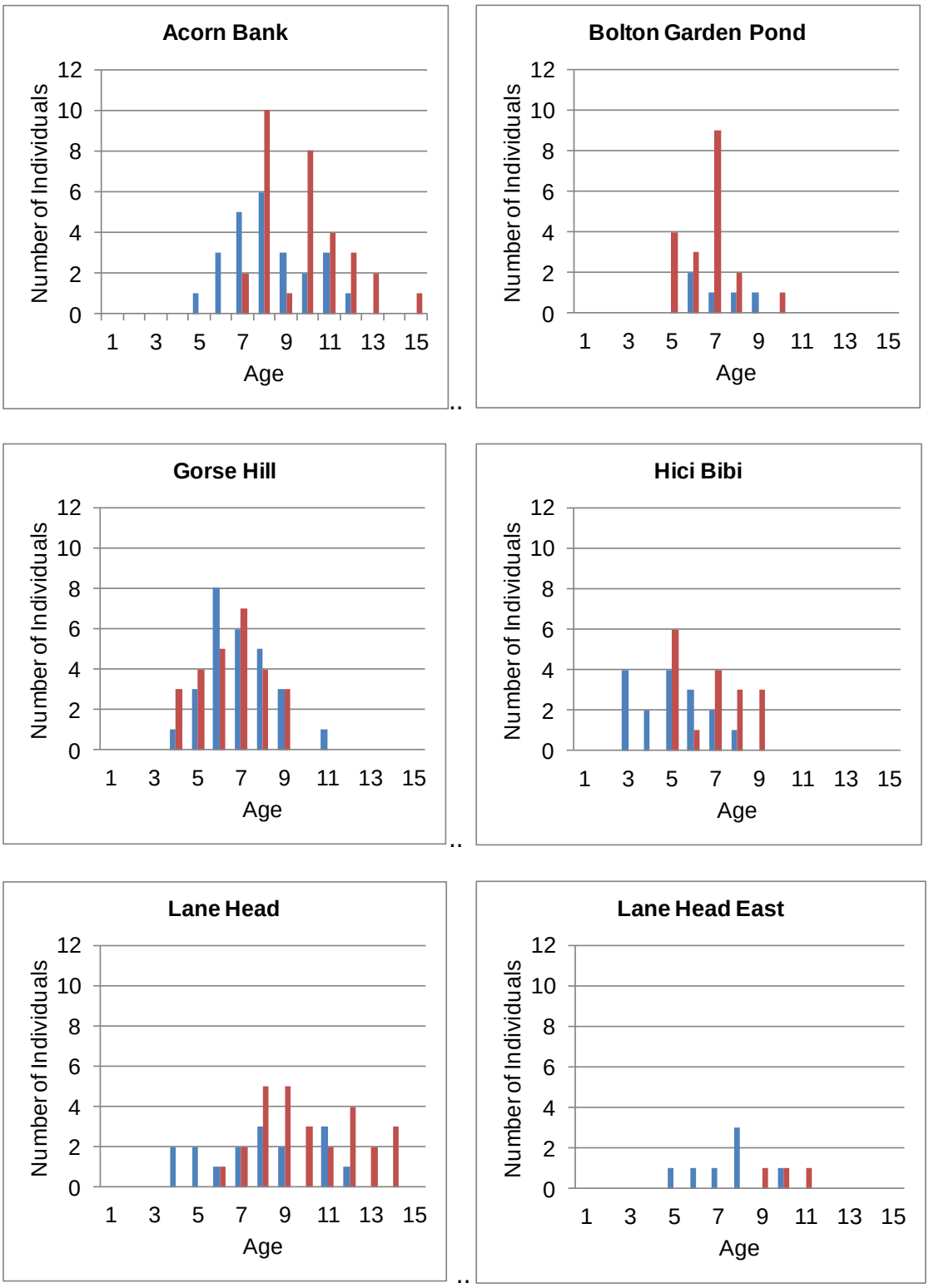


Figure 21: The estimated age of individuals in each population (Males shown in blue, females in red).

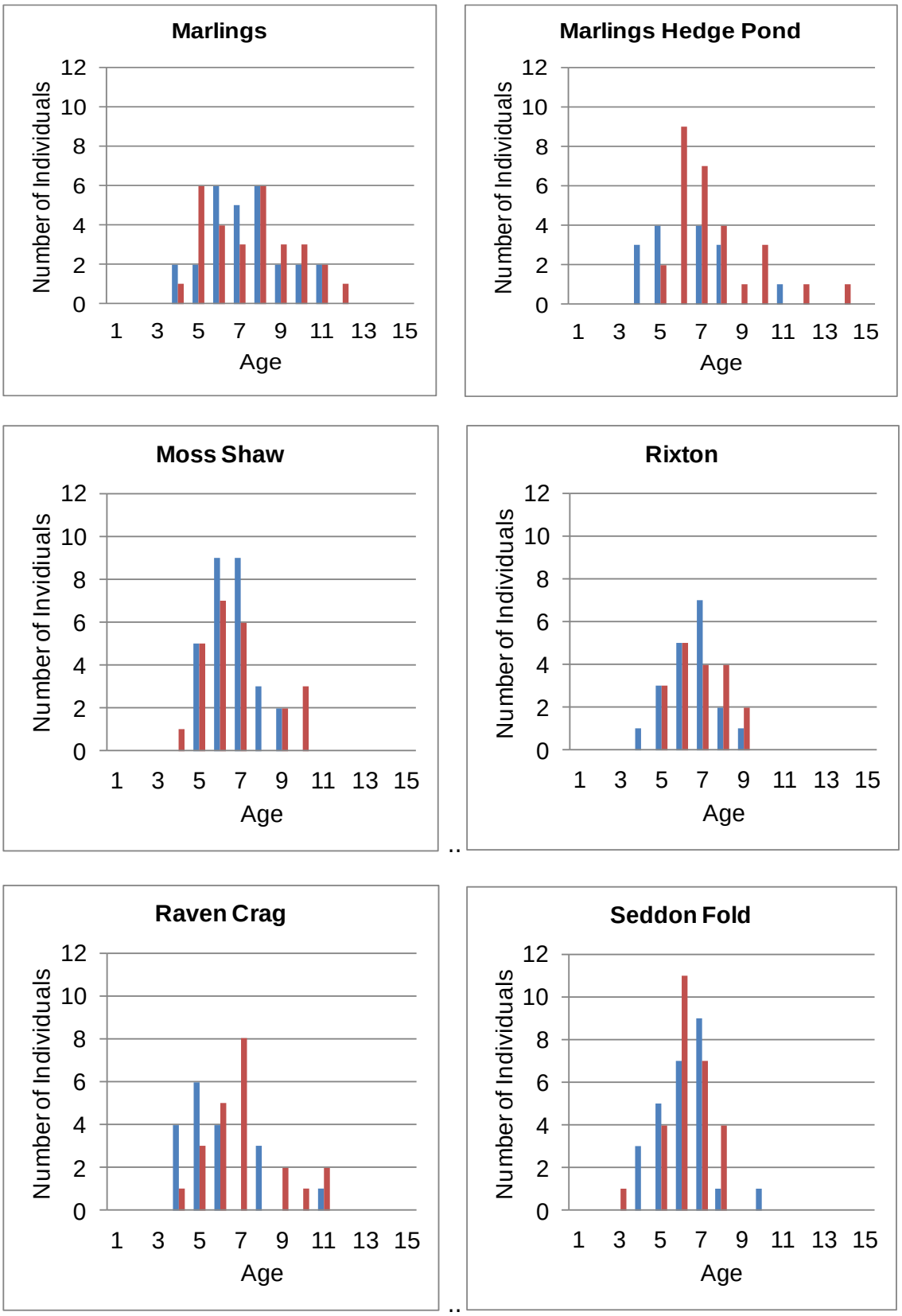


Figure 21: The estimated age of individuals in each population (Males shown in blue, females in red).

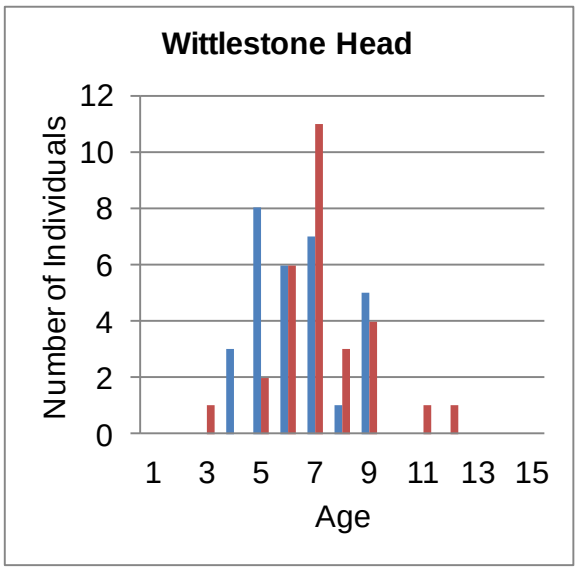


Figure 21: The estimated age of individuals in each population (Males shown in blue, females in red).

4.3.2 Comparison of the body condition of individuals at farmed and non farmed sites.

The longest individual was 94 mm in SVL (weighing 17g) and the heaviest was 22g (SVL 80mm). Both were females captured at LH. The longest individual for which age data are available is also the heaviest (92mm and 20.1g) captured at Bgp aged 8 years (sexually mature at 3 years). The mean length of adults was 72.1mm, and the mean weight was 8.6g. Overall, there is a consistent relationship between weight and SVL (Figure 22).

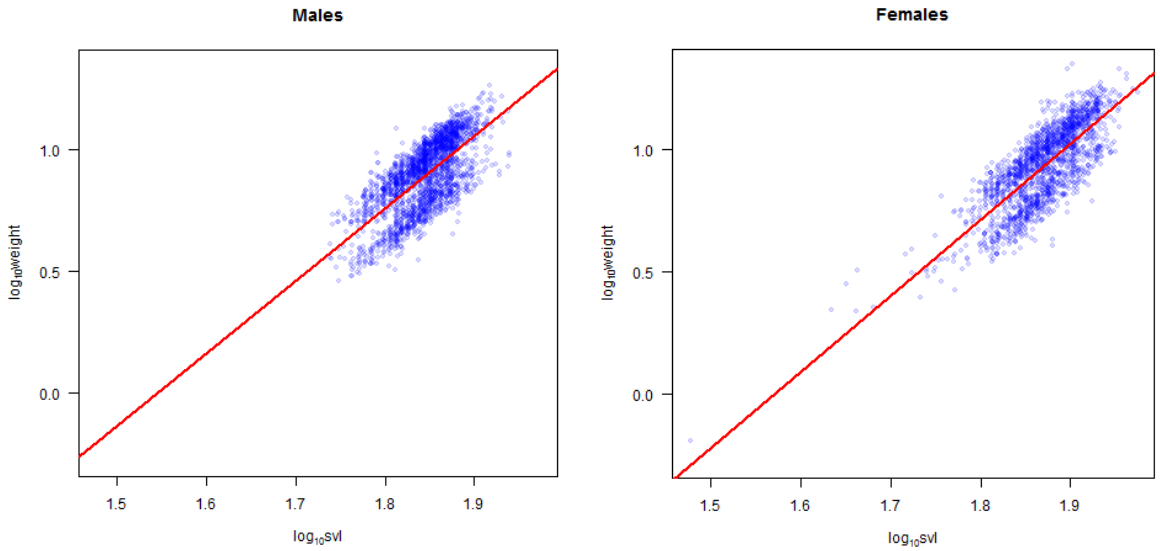


Figure 22: The relationship between log10 weight and log10 length for all males captured 2014-2016 (left) and all females (right).

Table 13 shows the median BCI values at each site. The distribution of BCI for all individuals weighed and measured is shown in Figure 23. Individuals with lower (negative) scores were more widely distributed than those with higher (positive) scores. BCI scores widely ranged between populations, however without apparent differences between populations at farmland and populations at other sites (Figure 24). Pairwise populations with significant differences in BCI between them are shown in Table 14.The was no significant relationship between BCI and population size for males ($p = 0.73$) or females ($p = 0.88$).

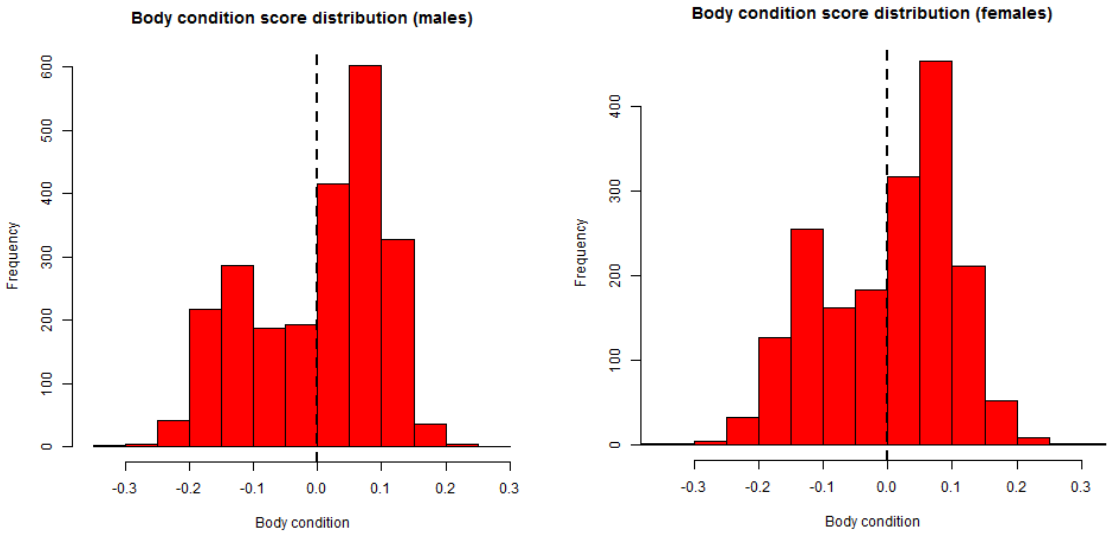


Figure 23: BCI for all males captured 2014-2016 (left) and all females (right)

Table 13: Summary of median male and female BCI at each site.

Site	Male Median BCI	Female Median BCI
AB	0.02	0.02
Bgp	0.06	0.08
GH	0.05	-0.02
HB	0.03	0.02
LH	0.02	0.10
Lhe	0.09	0.03
LHn	0.11	-0.01
Mhp	0.05	0.06
Marl	-0.09	0.06
MS	0.05	0.08
RC	0.08	0.06
R	0.03	-0.02
SF	0.02	0.02
WH	-0.05	-0.05

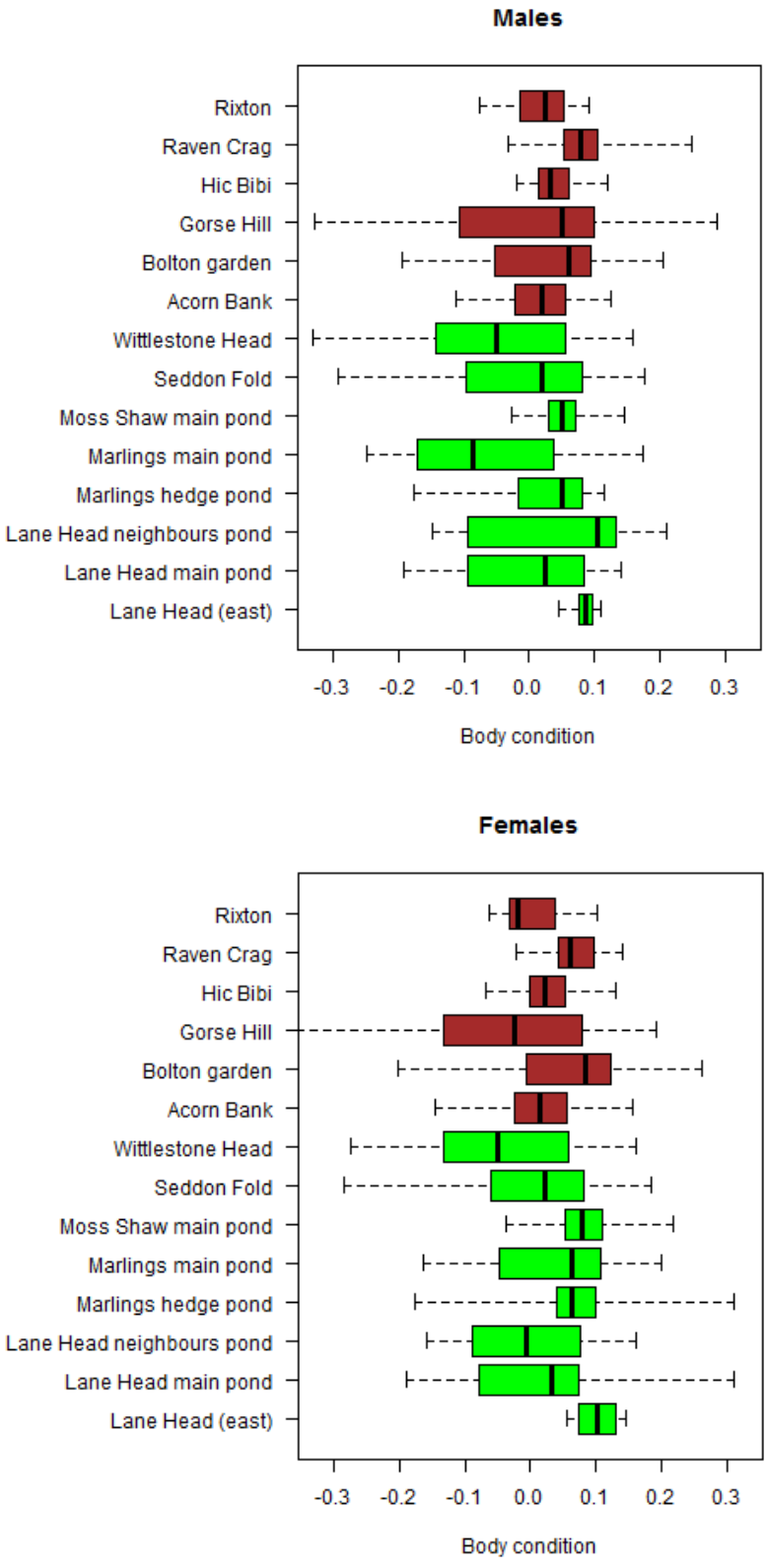


Figure 24: Box and whisker plot showing body condition for males and females across all sites. Green: farmed sites; red: non farmed sites.

Table 14: Kruskal-Wallis tests followed by a Dunn Test for multiple comparisons with Bonferroni correction, showing significant differences in BCI for pairwise comparisons. Only those comparisons with significant differences are shown.

Populations with significant differences in BCI.					
Males		p value	Females		p value
Marl	AB	7.86e-04	Marl	AB	7.86e-04
Marl	Bgp	3.13e-06	Marl	Bgp	3.13e-06
Marl	GH	1.99e-07	Marl	GH	3.13e-06
Marl	LHs	8.86e-04	Marl	LHs	8.86e-04
Marl	LHn	3.89e-04	Marl	LHs	1.54e-02
Marl	Mhp	5.00e-02	Marl	MS	3.24e-02
Marl	MS	2.69e-08	Marl	MS	2.69e-08
Marl	RC	4.99e-07	Marl	RC	4.99e-07
Marl	SF	4.18e-03	WH	AB	1.19e-05
WH	AB	1.19e-05	WH	Bgp	4.93e-07
WH	Bgp	4.93e-07	WH	GH	9.04e-17
WH	GH	9.04e-17	WH	LHs	3.11e-03
WH	LHs	3.11e-03	WH	LH	3.51e-02
WH	LH	3.51e-02	WH	LHn	1.30e-03
WH	LHn	1.30e-03	WH	MS	9.11e-10
WH	MS	9.11e-10	WH	RC	1.03e-06
WH	RC	1.03e-06	WH	SF	1.94e-03
WH	SF	1.94e-03	AB	MS	5.00e-02
AB	MS	3.24e-02	AB	RC	1.39e-02
AB	RC	1.39e-02	LHn	LH	3.89e-04
RC	SF	2.60e-02	RC	SF	4.18e-03
			RC	SF	2.60e-02

4.3.3 The effect of date of capture upon the age of individuals

Most individuals were toe clipped within the first 100 days of the year, since for most populations it was possible to toe clip 25 males and 25 females within several capture visits early in the season. However for small populations toe clipping continued throughout the season. This gave rise to the possibility that the date on which individuals were toe clipped could have biased the results of the study. If older individuals were more likely to return to the pond before (or after) younger ones they would therefore be over-represented in data. To investigate whether this could have occurred, the date of first capture is plotted against age in Figure 25. For both males and females, older individuals were captured earlier in the season than younger ones. Individuals aged 8 years and over were likely to be captured approximately 6 weeks prior to those aged 6 years. Individuals under 5 years, likely to be returning to breed for the first time, arrived at the pond approximately 3 weeks after the older newts, i.e. before those aged 6 years. Figure 20 also shows that males returned to the pond earlier than females of the same age. There was a significant difference between age classes with respect to their arrival time at the pond (males: Kruskal-Wallis chi-squared = 15.79, df = 4, p-value < 0.01; females: Kruskal-Wallis chi-squared = 26.94, df = 4, p-value < 0.01). The majority of individuals were toe clipped within the first 100 days of the year (Figure 26). Thus, most individuals aged 8 years and over were toe clipped at this time.

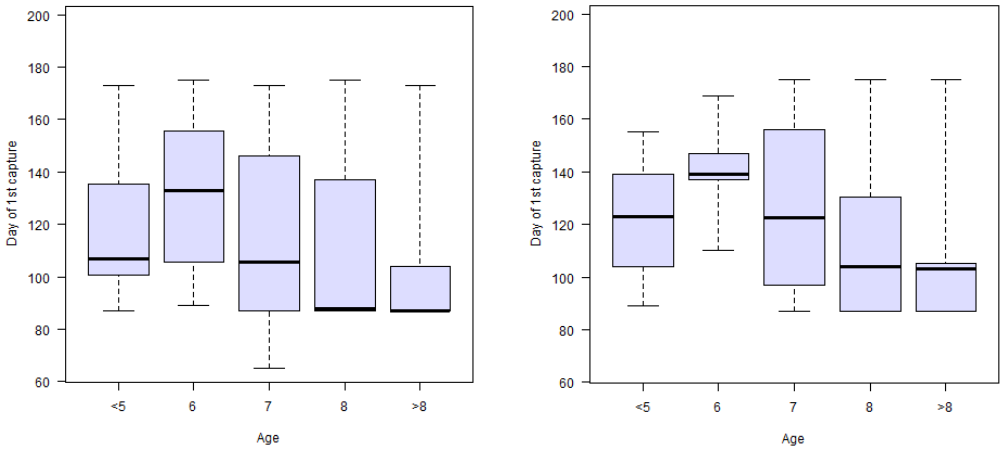


Figure 25: Age and date of first capture (left: males, right: females).

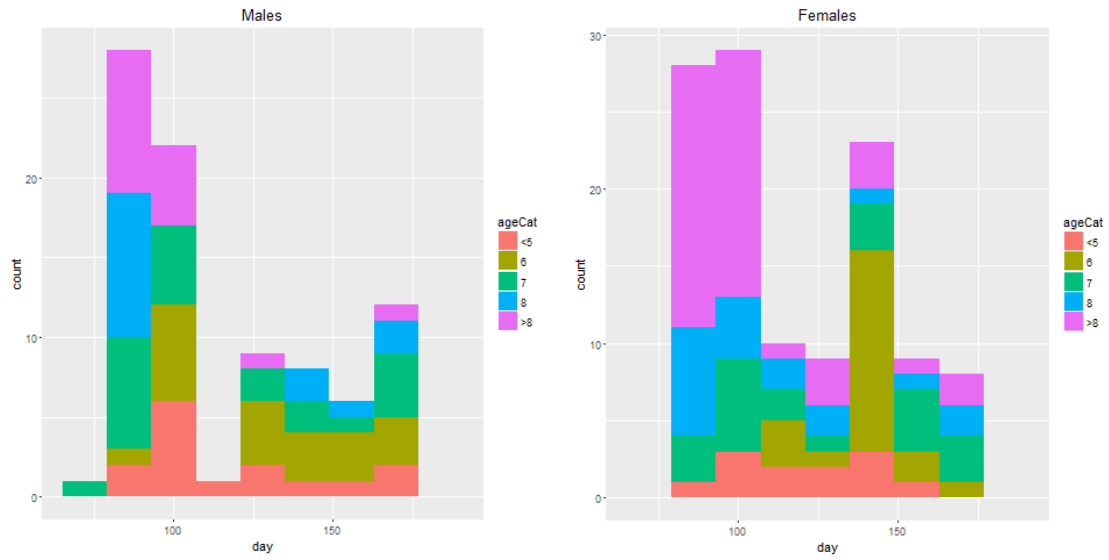


Figure 26: Time of the year that individuals of different ages were toe clipped. Age of individuals is represented by the different colours and Julian day is shown on the X axis.

4.3.4 Comparison of age with body condition

As age increases, BCI decreases in both sexes (Spearman Rank Correlation; p (males) = 0.02; p (females) = 0.01). Males below 5 years of age had a mean BCI of 0.06, which declined to 0.03 for those aged 8 years and over. Females of under 5 years had a mean BCI of 0.07, which declined to 0.05 in individuals aged 8 years and over (Figures 27 and Table 14).

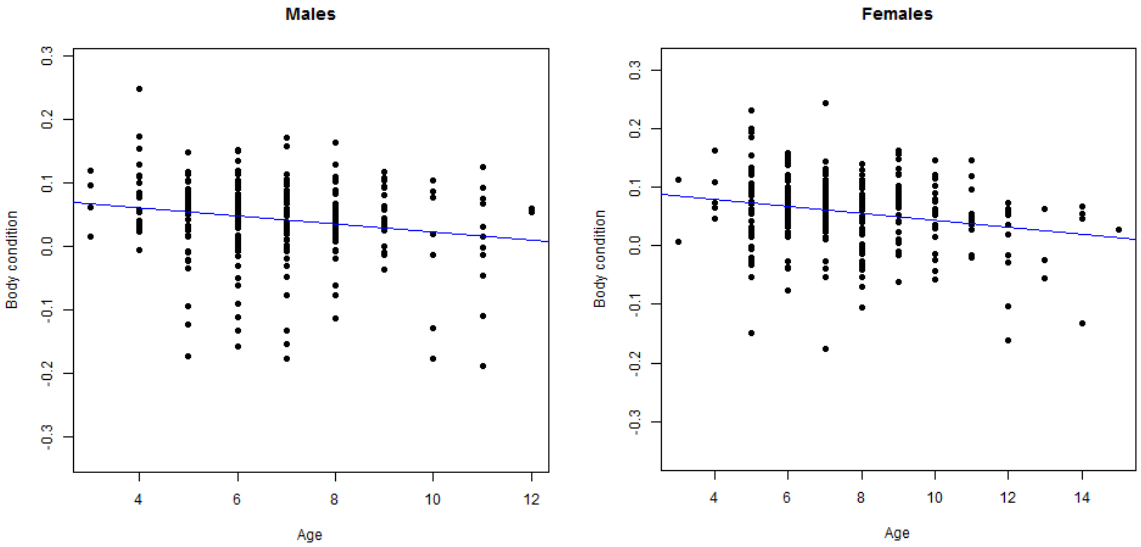


Figure 27: Comparison of age with body condition for males and females.

Table 15: Mean and median BCI of males and females of different age.

Age category	Males		Females	
	Mean BCI	Median BCI	Mean BCI	Median BCI
<5	0.06	0.06	0.07	0.07
6	0.04	0.06	0.07	0.06
7	0.04	0.05	0.07	0.07
8	0.04	0.04	0.04	0.04
>8	0.03	0.04	0.05	0.06

Comparisons between populations revealed marked differences in the relationship between BCI and estimated age for both males and females (Figures 28 and 29).

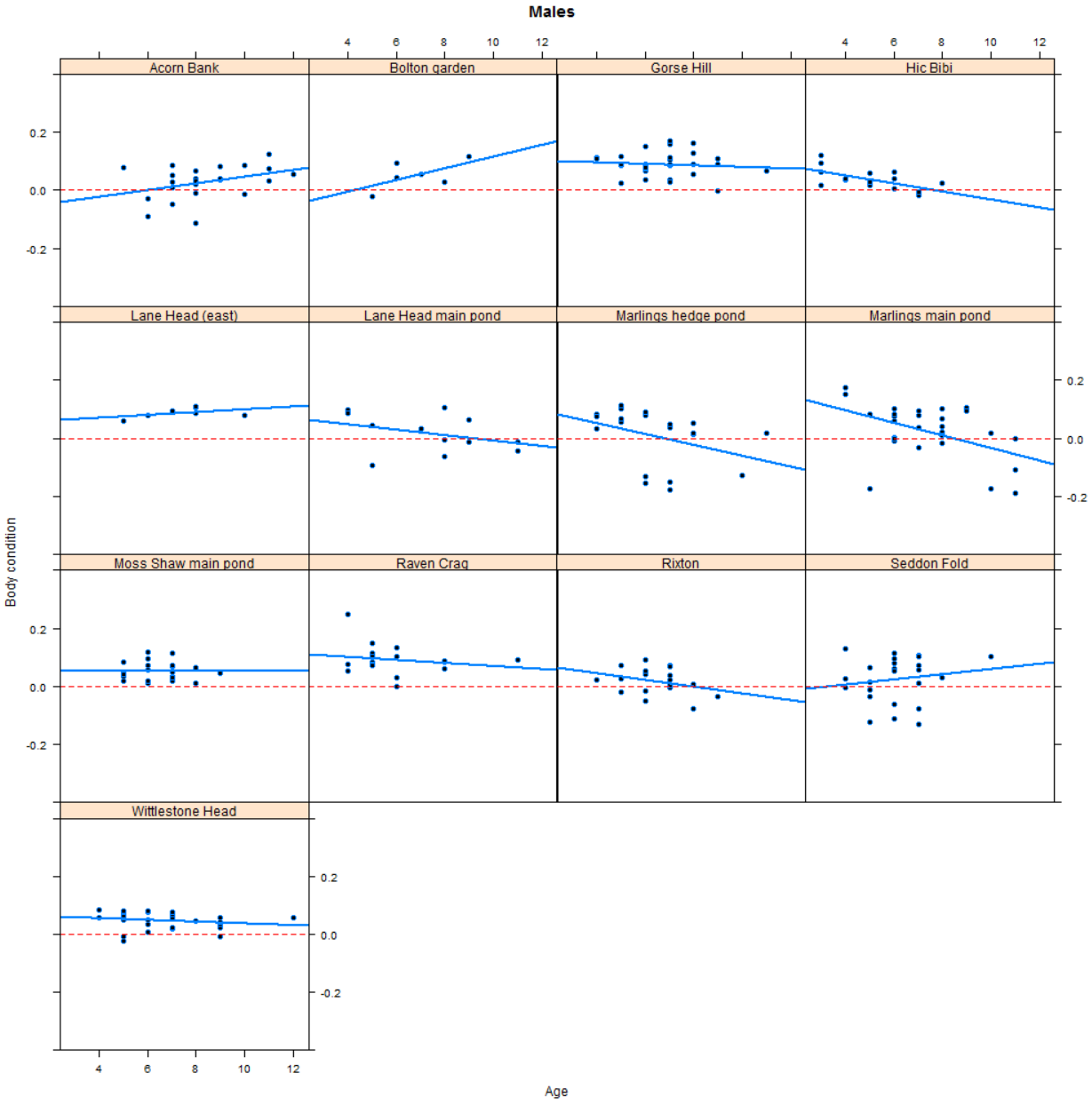


Figure 28: The relationship between male age and body condition at each study site. Across all sites, male BCI decreased with age but these graphs show the variability between populations, for example the garden pond showed a sharp increase in BCI with age.

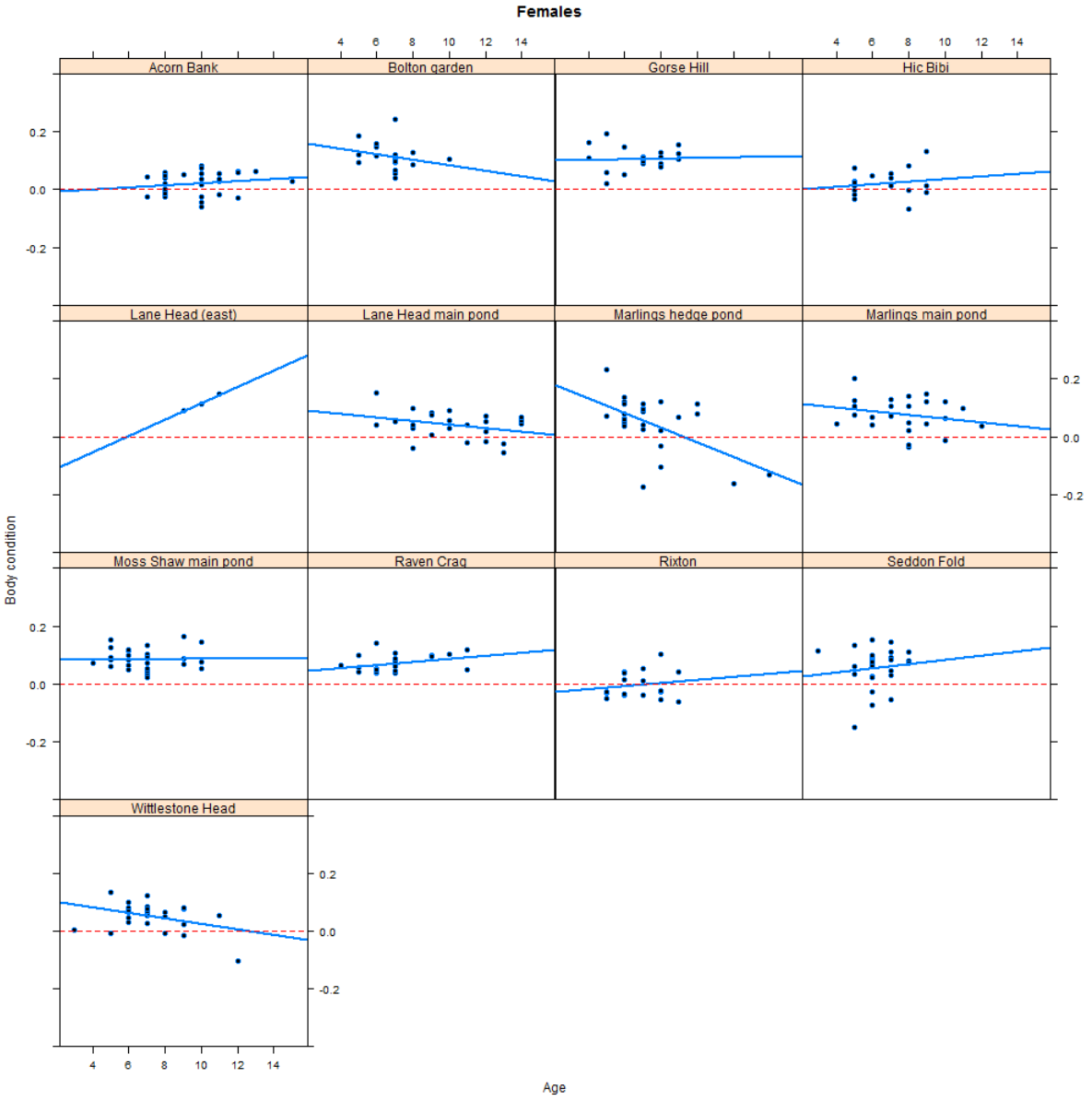


Figure 29: The relationship between female age and body condition at each study site. As with males, female BCI declined with age across all sites but there was considerable variation between populations. In contrast to the results for males, BCI for females at the garden pond decreased with age, This indicates a complex state of affairs suggesting that BCI is affected by different conditions at each site.

4.4 Discussion

Obtaining a Home Office licence to toe clip *T. cristatus* was difficult due to an absence of published studies demonstrating that the practice had no adverse effects on individuals. Although studies for other species are available (Phillott et al., 2008;

Ursprung et al., 2011) the Home Office was reluctant to accept that these were applicable to *T. cristatus*. Whilst in some cases the removal of a toe appeared to cause pain to individual newts, in other cases individuals showed no sign of pain, or did not react in any way to the amputation. During the study it was demonstrated that toes of *T. cristatus* regenerated quickly (Figures 14 and 15) and that individuals survived for at least 2 years after toe clipping.

4.4.1 Methodological considerations

Sometimes multiple LAGs may be formed, for example when periods of mild weather interrupt hibernation, and these rings risk being interpreted as annual growth rings. A further complicating factor is that in older individuals, endosteal resorption can take place which causes the inner LAGs to be lost (Castanet, 1975; Castanet & Smirina, 1990). This process starts before individuals reach sexual maturity, and all bones are therefore affected. Consequently the interpretation of the inner LAGs is prone to error and precise age estimates for individuals can only be provided up to about eight years of age (Sinsch, 2015). The ends of each toe bone are most affected by the resorption process, making them least valuable for skeletochronology work. Sections mid way along each toe bone are less affected and provide a more accurate indication of age. Despite these caveats, a comparison of age estimation using skeletochronology with known-age individuals confirms that this method is precise enough for demographic studies (Sinsch, 2015).

The counting of some LAGs was difficult as they were very faint and could have been overlooked, leading to an underestimate of age. However, Figures 20 and 21 show a large number of middle-aged individuals across all populations, which suggests that the toes have been aged consistently. Another source of bias is that toe samples were collected over 2 years. Out of a total of 548 toe samples, 379 were collected in 2014 and 169 were collected in 2015. All 53 samples from AB were collected in 2015, and additional collecting took place in 2015 at those sites where it was not possible to collect 25 male and 25 female toes in 2014 (for example Mhp) This complicates age comparisons between sites, which might not be fully representative for given years.

In the case of smaller populations, which did not provide 25 males and 25 females, collection took place across the entire sampling period. Other sites, which provided a large number of captures, required toe clipping on the first visits only. Two additional control sites (Raven Crag and Rixton) were visited only once near the end of 2014, providing 50 toes each. A study by Sinsch et al., (2003b) documents that *T. cristatus* individuals arriving at a pond early in the season are usually older than those arriving later. The study also indicates that younger individuals have a tendency to stay longer within the pond. Therefore, ponds which provided toes later in the season would misleadingly appear to consist of more younger individuals than those where toes were collected much earlier in the season. However, the age of newts at Lane Head, where toe clipping was carried out throughout 2014, shows a similar pattern of age structure to other sites, suggesting that the source of bias is unlikely to have affected the outcome of the study.

In some ponds it was possible to toe clip enough newts within several capture visits early in the season. The decreasing number of newts toe clipped over the season therefore reflects the decreasing number of toe clips taken during the season rather than the decrease of captures. The large proportion of older individuals captured during the first 100 days again demonstrates that older individuals have a tendency to return to the pond earlier in the season.

4.4.2 A comparison of the age structure of populations at farmed and non farmed sites.

The median estimated age across all populations (seven years) is reflected by few young as well as few old individuals recorded at most populations. The consistency of this pattern indicates a true reflection of population age structure, which is unlikely due to errors in the sampling or estimating process. Since a proportion of the population die each year, it would be reasonable to expect a large number of young individuals, gradually reducing in numbers with age. This pattern has been found in other amphibians, such as *Bufo calamita* (Sinsch, 2010). Diaz-Paniagua (1996) found a similar pattern in *Triturus marmoratus pygmaeus,* where the majority of individuals were estimated at two years old and the number of older newts

decreased until the maximum age of 10 years was reached. However, Olgun et al., (2005, with *Triturus karelinii*) found individuals to be sexually mature at 3-4 years, whereas the majority of males (n=19) and females (n=22) were estimated as six and five years old, respectively. The results therefore bore similarities to the present study and there are a number of possible explanations for this finding. Firstly, 2007 (i.e. 7 years before most samples were collected) could have been an excellent year for recruitment. Secondly, given the similar pattern of predominantly middle aged individuals at all sites, an environmental factor such as climate could be responsible for this observation. Thirdly, a large number of young individuals (3-5 year old) are indeed present in the pond but for some reason they are evading capture, either due to the date of capture visits or the methods used. Alternatively, although some individuals return to the pond to breed soon after becoming sexually mature, a large proportion may not return to the pond for a number of years. And finally, the age of sexual maturity could be higher than estimated using skeletochronology, and individuals therefore would not spend several years on land after becoming sexually mature. However, that the majority of individuals became sexually mature at either 2 or 3 years was also assumed by Baker (1998) for another study in England. Therefore, the most convincing explanation appears to be that sexually mature adults do not return to breed for a number of years. The capture of more younger males than females may indicate that males return to the pond sooner after reaching sexual maturity than the females.It may also reflect the findings of another study which found that annual survival was higher for males than females (Schwizer, 2007).

The oldest populations, with a median age of eight years, are Acorn Bank, Lane Head and Marlings. The oldest individual was a 15 year old female from AB. Within this population, none of the 53 individuals sampled was estimated at four years or below, and only one and two individuals were estimated at five and six years of age, respectively. Given a pond size of only 4m in diameter at a population size of 282 +/- 15 individuals (the fourth largest in this study), it is likely that the high density of adults has resulted in high predation of larvae and thus low recruitment.

The large number of older individuals at Lane Head is less easy to explain. Of the 37 individuals for which age was estimated, 29 were at least eight years old. The population size declined from an estimated 110 +/- 16 in 2013 to 50 +/- 5 in 2016, suggesting a lack of recruitment despite being in a pond rich farmed landscape with a high density of occupied ponds. As previously discussed, this could be due to predation of larvae by the high number of *Notonecta* in the pond. The assumption of a healthy population based on a large number of adults would therefore be unwarranted.

With a median age of five years, HB is the youngest population; out of the 33 individuals sampled, 16 were estimated to be aged five or under. The terrestrial habitat is favourable for *T. cristatus*, consisting of woodland, scrub and dense vegetation. Management work in 2008 improved the habitat quality of the site, and four new ponds were created. It is therefore possible that the relatively large number of young individuals has arisen from a recently expanded population.

4.4.3 A comparison of the body condition of individuals at farmed and non farmed sites.

Similar to age structure, BCI and size also varied markedly between populations and sexes. For example, females captured at LH were large (62.61% were over 80mm), and median female BCI was the highest of all populations. However, only 15.38% (n=78) of males at LH were over 80mm, and their median BCI ranked 10th among 14 populations. This contrasts with HB, where no males (n = 18) and only one female (n = 40) reached 80mm. Previous studies have indicated that the size of adults mainly depends on the size attained during the subadult growth period (e.g., Halliday & Verrell, 1988; Smirina, 1994). In the case of another amphibian species, *Bufo calamita*, adult size depended mainly on the size achieved between metamorphosis and first hibernation or aestivation (Sinsch, 2010). The large adults at LH could therefore be the result of a period of rapid growth earlier in their life histories.

Data on the length and age of individuals was considered to ascertain whether age of *T. cristatus* could be inferred from length. A previous study using 16 individuals (Hagstrom, 1980) concluded that it was not possible to assume that short individuals

are young. In this thesis, data in general terms show that short individuals are often younger than long ones. For example, 68 individuals measured at 60-65mm SVL had an estimated age of between three and nine years, with the majority being between four and six years of age. 102 individuals measured at 80-92mm SVL varied between 5 and 15 years of age, with the majority being between 9 and 11 years old. However, this study confirms that although smaller individuals are generally younger than longer individuals, age cannot be directly inferred from length.

BCI appeared to be unrelated to whether populations inhabited farmed and non-farmed sites. It would have been reasonable to expect that newts in the more structurally varied habitats, primarily those at GH and to an extent WH, would have a higher BCI than those at farm sites with little structural diversity, such as MS. This expectation is supported in study by Scheele et al., (2014) on the body condition of *Bombina variegata* in Romania which found that toads in forest ponds had significantly better body condition than those in pasture ponds. However, when GH was compared to the other populations a similar pattern was not found and in fact WH was the only site with negative BCI values for both males and females, with BCI of males and females being significantly different from those in nine and eight other populations, respectively. At 250m altitude, WH is located 100-150m higher than all other sites, possibly resulting in lower mean temperatures and relatively low BCI in both males and females. This finding reflects one of the observations from a previous study by Ficelota et al., (2010) of a strong geographical variation in body size of *Triturus carnifex*. The study also noted that larger body size of *T. carnifex* was associated with colder climates. Whilst the relatively small increase in altitude at WH is unlikely to be reflected in a discernible increase in body size, it is possible that the higher altitude is manifested in the BCI of this population. According to the measure of BCI, *T. cristatus* generally does not appear to be adversely affected by farming practices.

4.4.4 The effect of date of capture upon the age of individuals

This study showed that older individuals were captured earlier in the season than younger ones, but that individuals under 5 years, likely to be returning to breed for the first time, arrived before the majority of newts, aged 6-7 years. Reasons for this are open to speculation but may be related to body condition (discussed below).

4.4.5 Comparison of age with body condition

BCI decreased with age, a trend which was more pronounced for males than females. This finding could be related to the data showing that individuals aged 8 and over (n=197) returned to the pond before younger individuals. It could also be an effect of ageing and senescence. *Triturus cristatus*, like some birds and mammals, may slow down their reproductive activity with age. Assuming that younger individuals have a higher BCI because they have higher fat reserves relative to their length, they would be better prepared for the mating season than their older counterparts, combined with less urgency to return to ponds early in the breeding season. Arntzen (2000) observed that growth rate diminished with size, and it has been suggested that older individuals allocate smaller amounts of energy into growth relative to reproduction (Czarnoleski and Kozlowski, 1998). Large size in urodeles is associated with female fecundity (Sullivan et al., 1998), and investment in early reproductive activity may generally reduce growth and diminish lifetime reproductive success (Stearns, 1992). This would favour delayed maturation and large body size for females (Arntzen, 2000), and could explain the apparent lack of younger individuals found in this study. The absence of a clear relationship between male size and mating success (Hedlund, 1990; Green, 1991) and the relatively low reproductive cost could explain why more younger males than females were found in most populations in this study. In this scenario, those individuals aged 6 and 7 may return to the pond later because they are still preparing for breeding activity and/or because they do not have the energy reserves to sustain them for the entire breeding season. The present study was not able to provide data on time spent at ponds, but he youngest individuals might have compensated for their relatively early arrival by leaving sooner.

Chapter Five: Genetic structure and the effects of isolation on *T. cristatus* in the farmed landscape.

Summary

Data derived from genetic markers such as microsatellites make it possible to assess the effects of farming on the spatial structure of *T. cristatus* populations. A total of 715 samples from 23 ponds on seven sites were genotyped with seven loci. Levels of genetic variation were high overall, but with only moderate links between numbers of alleles per locus and observed heterozygosities. Measures of genetic distances (measured through F_{st}) were low to moderate, suggesting that populations were at least historically connected. Patterns of isolation-by-distance were most significant for comparisons of ponds within sites and were not significant for comparisons of ponds between sites only, reflecting that gene flow is impeded through fragmentation at the scale of the entire study area. Overall, the results suggest that the study populations are largely genetically healthy, although connectivity between ponds needs to be maintained or improved to ensure their long-term survival.

5.1 Introduction

Agricultural intensification, changes in farming practice and pond loss have led to a widespread decline in habitat quality for amphibians (Jeliazkov, 2013). This may have long-term impacts on the status of species due to the loss of genetic diversity. Evidence of this could affect future conservation of the species therefore correctly estimating long-distance dispersal is essential to determining the appropriate scale of a metapopulation approach (Smith & Green, 2005). Newts have been found at high densities in terrestrial habitats up to 200 m away from a breeding pond (Franklin, 1993) and previous studies have estimated a maximum migratory range for *T. cristatus* as 250 m from a pond (Oldham and Nicholson, 1986, Franklin, 1993 and Jehle, 2000). More recently *T. cristatus* has been found to move up to 1.3 km between breeding ponds and up to 1.6 km over a 75 day period (Haubrock & Altrichter, 2016).

Genetic studies using microsatellite markers allow metapopulation dynamics at the regional scale to be investigated, including questions of source-sink dynamics and population connectivity. It is now possible to classify individuals according to the most likely population of origin, based on their genotype (Rannala & Mountain, 1997; Waser & Strobeck, 1998; Dawson & Belkhir, 2001). Using a sufficient number of variable loci in combination with an adequate sample size the approach is surprisingly powerful, even when the reference populations are genetically rather similar (Bernatchez & Duchesne, 2000). A very useful application of assignment methods for conservation-related research on amphibians lies in measuring between-population connectivity at a scale equal to, or smaller than, the migratory range of the relevant species. Work conducted on *T. cristatus* in Flanders (Northern Belgium) indicated that dispersal and migration rates are limited at the geographic scale, but that habitat fragmentation had not yet led to a significant loss of genetic diversity. This could be because individuals are relatively long-lived, fragmentation of their habitat is relatively recent in Flanders, and most ponds in the study are still connected at the local scale (Schon et al., 2011).

Movement distances detected by tracking or C-M-R studies are usually far below the corresponding estimates based on gene flow data. Sinsch (2014) stated that this discrepancy reflects the constraints of available tracking methods for free-ranging individuals leading to underestimates of annual movement. He came to three conclusions regarding movement of individuals and the genetic structure of adjacent amphibian populations: (i) individual movements, or a consecutive series of movements, can lead to misleading under estimates of total movement capacity; (ii) modelling of probable movement capacity is the best available predictor of gene flow between adjacent populations; (iii) connectivity of populations is less affected by landscape resistance than previously expected. Given the practical difficulties of tracking *T. cristatus* in its terrestrial habitat, genetic techniques are most suited to achieving the aims of this research. The ability of *T. cristatus* to disperse across the agricultural landscape was addressed by investigating spatial distribution of genetic variation in a study utilising microsatellites. Allele frequency is a measure of genetic variation giving a measure of expected (H_e) and observed (H_o) heterozygosity in a population.

The study was conducted using seven microsatellite loci to genetically characterise populations from 23 ponds on seven sites. This enabled comparisons between neighbouring ponds, and to determine the extent of gene flow between them. Where possible, a number of populations from each site have been sampled allowing comparisons not only between ponds on the same site but between sites which are separated by up to 41 km. The design of the study, which enables populations to be compared at different spatial scales, has been undertaken for other amphibian species in the UK such as Rana temporaria and Bufo bufo (Brede & Beebee, 2004) and Bufo calamita (Rowe et al., 1998 and Rowe & Beebee, 2007) but until now this has not included *T.cristatus*.

5.1.1 Research objectives

This study had three objectives (i) To find out whether individuals were able to disperse between ponds within the modern agricultural landscape. (ii) To ascertain whether isolation had any measurable or adverse genetic effects on *T. cristatus* populations and (iii) To ascertain the success of a *T. cristatus* introduction.

5.2 Materials and Methods

5.2.1 Fieldwork

Fieldwork was conducted in the spring of 2013 and 2014 when genetic material was collected in the form of *T. cristatus* eggs at the tailbud stage. This avoids potential injury caused by buccal swabbing and the risk of insufficient DNA collected by skin swabbing. Genetic material was collected from the 23 ponds shown in Table 16, the locations of which are shown in Figure 30. The objective was to collect thirty eggs per pond and a maximum of ten eggs per visit to minimise the risk that eggs from the same female were collected. Samples were taken on at least three different visits from various places along the pond edge. Samples were stored in 70% alcohol with approximately 10 eggs per tube. The only pond at which it proved impossible to find eggs was Seddon Fold Farm, where four years of C-M-R studies had been undertaken

Table 16: The number of genetic samples collected per pond.

Study site	Samples collected
AL	17
Bgp	37
BFi	20
BFt	34
GH	60
GHj	29
HB	45
HBd	48
HBs	22
LH	77
LHs	31
LHsd	30
LHss	20
Marl	17
Mdsp	40
Mgp	22
Mhp	72
MR	10
MS	67
MSmud	13
MSsp	23
WH	57
WHR	37
Total	**828**

Figure 30: The location of sites included in the genetic study. Population estimates and age structure are available for ponds in red, age structure estimates only are available for ponds in yellow.

5.2.2 DNA extraction and PCRs

DNA extraction was carried out following a phenol-chlorophorm protocol used by Jehle et al., (2013). Seven microsatellite markers were selected for amplification following Krupa et al., (2002): *Tcri* 13, 27, 29, 35, 36, 43 and 46. They were modified with fluorescent dyes FAM, AT550 or HEX. Due to a high failure rate of PCR

reactions using primer 36, this locus was however substituted with primer 50 (Drechsler et al., 2013) in the course of the study. A Polymerase Chain Reaction (PCR) was used to amplify the seven microsatellites as outlined in Krupa et al., (2002, for further information see Table 18). A Veriti 96 Well Thermal Cycler (ABI) was used to conduct PCRs of 10 µl reaction volumes. Each 10 µl reaction volume contained 1 µl of genomic DNA, 0.1 µl dNTPs (2mM), 0.1 µl of forward and 0.1 µl of reverse primer (2 mM), 0.1 GoTaq® (NH4)2SO4, 670 mM Tris-HCl (pH 8.8 at 25° C), 0.1% stabilizer) and 7 µl of PCR grade H_2O. The temperature profile used was that described by Krupa et al., (2002): 94°C, 2 min, 1 cycle, then (94°C, 30 s, T*m*°C, 30s, 72°C, 30 s) for 39 cycles; T*m* varied between 50–56°C depending on the locus. Touchdown PCRs were based on the above profile, except that the annealing temperature was dropped by two degrees from 64–56 °C after two cycles at each temperature, followed by 22 cycles at 55°C.

Amplified PCR products were detected visually by electrophoresis before genotyping, allowing the success of reactions to be assessed. Gels of a 1.5% agarose concentration were prepared using a solution of 1.5 gram of agarose powder (Bioline Ltd, UK) to 100 ml of 0.5x Tris-borate-EDTA (TBE) buffer (89 mM Tris-borate, 2mM EDTA, pH 8.3, Severn Biotech, UK). 100µl of GelRed™ agent (Biotium, Hayward, CA, USA) was mixed into the solution. GelRed™ is an intercalating agent which binds to the DNA, fluorescing under UV light for visualisation. Between 3 µl and 5 µl of PCR products were mixed within 4µl of loading dye in separate PCR tubes, ensuring the original PCR products were kept for future genotyping. A mix of 5 µl of Hyperladder II, 1kb or 100bp and 4 µl loading dye was loaded consistently into the first well and the PCR products were then loaded into the following wells. The solidified gel was fully submerged in an electrophoresis bath containing TBE buffer solution. Electrophoresis ran for 1 hour at 110 volts, between 70 and 100 mA. A transilluminator was used to visualise the DNA molecules within the gels. Photographic evidence was taken using the software Genesnap from Syngene and printed copies of pictures were created with a G-Box Syngene (an example is shown in Figure 31). From the 828 samples collected (Table 16), products were obtained for a total of 715 samples using primers *Tcri* 13,

27, 29, 35, 43, 46 and 50 (Table 17). Population size estimates are available from eight of these ponds (see chapter 3).

Table 17: The number of successful PCR reactions per pond. Sites with population estimates are in bold.

Pond name	Successful PCR reactions
AL	16
Bgp	36
BFi	14
BFt	31
GH	52
GHj	26
HB	40
HBd	42
HBs	14
LH	70
Lhs	28
LHsd	19
LHss	15
Marl	13
Mdsp	37
Mgp	22
Mhp	63
MR	8
MS	57
MSmud	11
MSsp	20
WH	48
WHR	33
	715

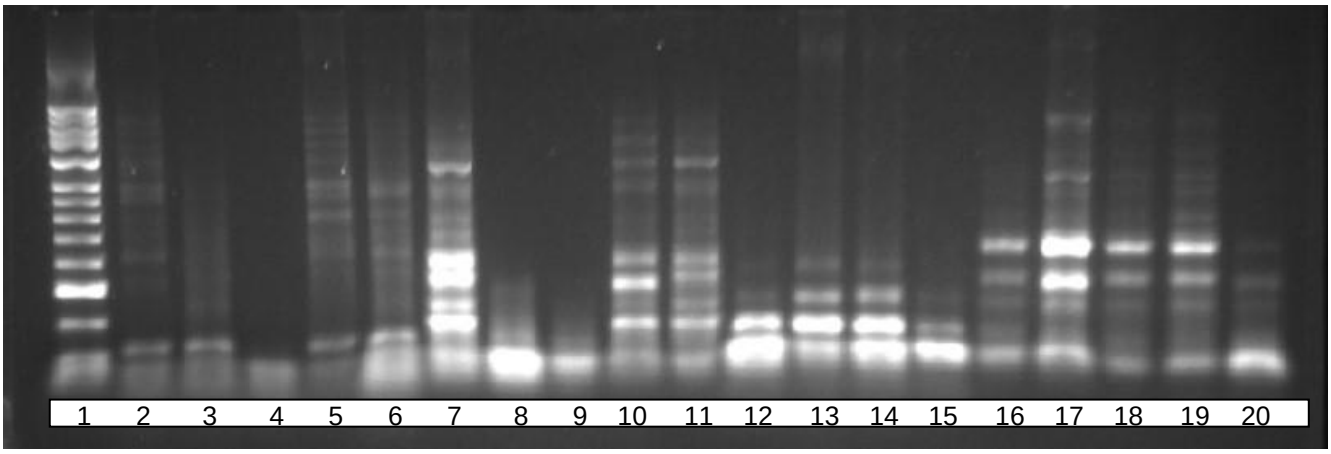


Plate and primer	#	Plate 6 Primer 13					Plate 6 Primer 29					Plate 6 Primer 35					Plate 6 Primer 46			
Gel well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Result	#	M	M	F	M	M	S	F	F	S	S	W	S	S	W	M	S	S	S	M

Figure 31: Example results of a gel electrophoresis conducted on 27.7.15. The Hyperladder is on the left and lanes have been annotated to correspond to an extract for the gel record sheet. Gel results for primer 13, 29, 35 and 46 are shown. Bands have been classified as weak (W), medium (M), strong (S) or fail (F).

Table 18: Characteristics of microsatellite markers used in this study as described by Krupa et al., (2002; 13-46) and Drechsler et al., (2013; 50). Note: the size range shown for each primer is a combination of that given in both papers. This allowed a larger range for each product, a precautionary measure to ensure no confusion arose from multiplexing up to four PCR products.

Locus		Primer sequence	Size range of amplification product	Number of alleles	EMBL accession	Primer dye	Annealing temperature
*Tcri*13	F:	GTGATGGTTGCCAAGC					
	R:	GATCCAAGACACAGAATATTTAG	93-131	4	AJ292500	FAM	55°C
	F:	GATCCACTATAGTGAAAATAAATAATAAG					
*Tcri*27	R:	CAAGTTAGTATATGATATGCCTTTG	241-295	6	AJ292517	HEX	50°C
	F:	CGAGTTGCCCAGACAAG					
*Tcri*29	R:	GATCACATGCCCATGGA	289-340	5	AJ292505	FAM	55°C
	F:	CCAACTGGTATGGCATTG					
*Tcri*35	R:	GATCACAGAAACTCTGAATATAAGC	185-234	5	AJ292490	HEX	55°C
	F:	CTTTTCACACCACTGGAGCA					
*Tcri*43	R:	GTTTCTATTAGTCTGGCATTGGCTGC	262-298	9	AJ292511	AT550	50°C
	F:	CAAGTTTCCTCTGAAGCCAG					
*Tcri*46	R:	GTTTCTTGCCTGACAAAGTAATGCTTC	253-311	6	AJ292494	AT550	50°C
	F:	F: GCGGATACATGGTCTTCGTT					
*Tcri*50	R:	R: TTCAGTTAAAAGTGTCCTCTGTGG	177-268	26	KF442195	AT550	60°C

5.2.3 Genotyping

Due to the high sensitivity of the genetic analyser (Applied Biosystems Ltd.), DNA concentrations of each PCR product were diluted using distilled H_2O. Band strength and primer dye were used as the basis for dilution rates of each PCR product as shown in Table 19. Dye AT550, the weakest dye, was diluted the least and FAM, the strongest, was diluted the most. Band strengths defined as faint were diluted least, with no dilution for AT550.

Table 19: Dilution rates used for the primer dye.

Dye	FAM	HEX	AT550
Band strength: Weak	1:10	1:5	No dilution
Band strength: Medium	1:50	1:25	1:5
Band strength: Strong	1:100	1:50	1:10

Diluted PCR products were multiplexed in 96 well genotype plates. Two plates were used for all seven PCR products from each site. Primers 13, 27, 29 and 46 were multiplexed together, as were primers 35, 43 and 50. Genotype plate maps of 96 wells were used to record the transfer of samples from labeled PCR tubes.

A master mix volume of 9 µl containing 0.1 µl of size standard LIZ , 5 µl formamide and 3.9 µl of ddH_2O was loaded into a new 96 well genotyping plate. A multichannel pipette was used to transfer 1 µl of the multiplexed diluted PCR products into corresponding wells. A sterile septa cover was placed over the plate and the products were denatured for 10 minutes at 95°C before being put directly on ice. A further 10 minutes was used to cool the products. The plate was then loaded onto an ABI3130 Genetic Analyser for genotyping.

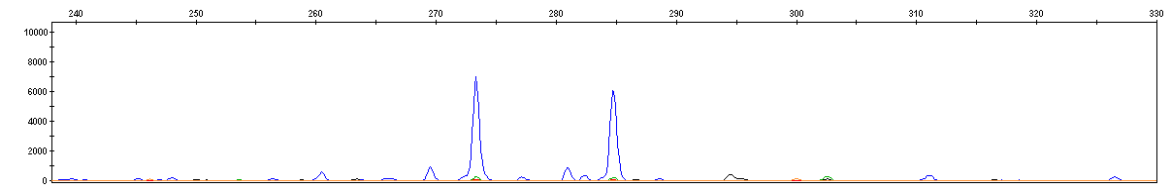


Figure 32: Example of allele size scoring at WH. The above screenshot is for primer 43, showing a heterozygous individual with base pairs at 272 and 284.

The genotypes were size scored using the programme Genemapper and the result interpreted manually to ascertain the base pairs for each allele. Figure 32 shows an example (peaks indicating alleles 272 and 284 basepairs in length). Both alleles are preceded by smaller peaks, referred to as stutter peaks. The size scored data were converted into a text file and analysed using the programme Genepop on the Web version 4.2 (Rousset 2008) to perform tests for Hardy Weinberg Equilibrium (HWE), and to calculate allele frequencies and pairwise fixation indices (F_{ST}). Isolation-by-distance was tested using the software IBD to correlate F_{ST} and log-transformed geographic distances. Geographic distances to the centre of each pond were calculated to the nearest 5 m using Google Earth. Partition of genetic variation among populations was addressed using BAPS v.6 (Corander et al., 2004, Cheng et al., 2013). BAPS treats allele frequencies and the number of genetically divergent groups in a population as random variables. It runs a mathematical algorithm over genetic data in which each individual genotype has a pre-defined assignment to a given number of populations (in this case ponds) to determine which of these populations are also meaningfully represented as genetic units (“clusters”). Those populations which bear distinct genetic signatures are represented as a single cluster, whereas ponds which are genetically rather similar become merged into shared clusters.

5.3 Results

Of the 828 samples collected for which PCR products were obtained, 113 were excluded from the final analysis because they had fewer than three successful PCRs. A total of 715 samples from 23 ponds on seven sites were successfully size scored. A summary of the results is shown in Table 20. Mean number of alleles per locus and observed heterozygosity, ranked lowest to highest, are shown in Table 21. Allele frequencies are shown in Appendix 8.

Table 20: Descriptive population genetic data for the 23 populations. H_o: observed heterozygosity, H_e: observed heterozygosity, *HWE*: Hardy-Weinberg equilibrium.

Pond	mean number of alleles/locus	H_e	H_o	Loci out of HWE
AL	7.33	0.80	0.83	
Bgp	5.33	0.64	0.64	27,35,43
BFi	6.83	0.78	0.82	27,50
BFt	8.14	0.81	0.86	13,35,43
GHj	6.86	0.76	0.88	27,29,35,43,46
GH	7.29	0.75	0.83	46,50
HBd	8.86	0.80	0.88	29,35,46,50
HB	7.17	0.74	0.89	13,27,43,46
HBs	6.33	0.80	0.87	
LH	8.57	0.77	0.84	46
LHs	6.83	0.75	0.75	50
LHsd	4.67	0.58	0.72	35,50
LHss	4.17	0.65	0.87	13,46
Mdsp	8.14	0.75	0.78	27,29,35
Mgp	5.29	0.72	0.66	50
Mhp	7.86	0.72	0.76	29,35,50
Marl	6.57	0.76	0.76	43
MR	5.17	0.76	0.88	
MS	9.86	0.82	0.85	35,43
Msmud	5.57	0.77	0.75	43,50
MSs	6.5	0.79	0.89	50
WH	6.14	0.65	0.75	35,43,46
WHR	9.5	0.81	0.83	

Table 21: Mean number of alleles per locus and observed heterozygosity for each site, ranked from lowest to highest. A Spearman Rank Correlation coefficient of 0.29 shows a weak positive but non significant (P = 0.19) correlation between the two sets of values.

mean number of alleles/locus		*Ho*	
4.17	LHss	0.64	Bgp
4.67	LHsd	0.66	Mgp
5.17	MR	0.72	LHsd
5.29	Mgp	0.75	LHs
5.33	Bgp	0.75	Msmud
5.57	Msmud	0.75	WH
6.14	WH	0.76	Mhp
6.33	HBs	0.76	Marl
6.5	Mss	0.78	Mdsp
6.57	Marl	0.82	Bfi
6.83	BFi	0.83	AL
6.83	LHs	0.83	GHm
6.86	GHj	0.83	WHR
7.17	HBm	0.84	LHm
7.29	GHm	0.85	MS
7.33	AL	0.86	BFt
7.86	Mhp	0.87	HBs
8.14	Mdsp	0.87	LH
8.14	BFt	0.88	GHj
8.57	LHm	0.88	HBd
8.86	HBd	0.88	MR
9.86	MSm	0.89	HBm
9.5	WHR	0.89	MSs

The Hardy Weinberg Equilibrium (HWE) is the balance between expected and observed heterozygosity based on allele frequencies. Only four of the 23 populations did not show significant deviation from the HWE at any locus. As the deviations are not based on specific loci, it is unlikely that they are caused by locus-specific non-amplifying alleles. H_e is higher than H_o in 18 out of 23 populations.

There was a large variation in the total number of alleles found per locus, from 16 at locus 35, to 31 at locus 50. The mean number of alleles per locus for each population ranged from a minimum of 4.17 at LHss to 9.86 at MS. The largest populations LH and MS were characterised by 9.86 and 8.57 alleles per locus,

respectively. This was more than the smaller populations on those sites. However Marl, the largest of five populations sampled, had a mean number of 6.57 alleles per locus. This was higher than MR and Mgp, which are likely to be small populations, but lower than Mdsp and Mhp, both of which inhabit smaller populations than Marl. (During fieldwork, only two individuals were captured at Mdsp therefore it was not possible to calculate a population estimate at this pond.) This shows that populations with the highest number of mean alleles per locus are not necessarily those with the largest populations. Bgp, the introduced population, did not have the smallest mean number of alleles relative to other populations in this study. Populations with fewer alleles per locus (AL, LHss, LHsd, Mgp, and MR) were all identified as being small.

In addition to the number of alleles per locus, genetic variation can also be expressed as allele frequencies. Five of the six populations with the largest amount of genetic variation (>8 alleles per locus) are all from ponds within the agricultural landscape. The populations with the smallest number of alleles per locus (<6) were those where only small numbers of eggs were found (LHsd, LHss, MR, MSmud) or where the populations were small (Bgp and Mgp). In all but one of these ponds (Bgp) only a small number of individuals (<5) were captured during trapping visits.

5.3.1 Investigation into the dispersal of individuals between ponds in the modern agricultural landscape.

F_{ST} is a measure of genetic distance, or differentiation. Both genetic and geographical distances between ponds are shown in Table 23. Populations with values close to 0 are less genetically differentiated than those with higher values. There is a low level of differentiation between some ponds on the same site, such as Marl and Mhp with a value of 0.01. However, small F_{ST} values are also obtained when comparing populations on different sites, eg LH and BFi (0.03) and LH and BFt (0.07). The software IBD enabled a comparison of the genetic distance between ponds, produced by Genepop, with the geographic distance between ponds. A matrix showing the distance between ponds measured in Google Earth is shown in Table 22. Isolation by distance results from IBD are presented in Table 23 and in Figures 33-35.

Table 22: Genetic distance (F_{ST}) between ponds (top right half of the table) and geographic distance (m) between ponds (bottom left).

	AL	Bgp	BFi	BFt	GHj	GH	HBd	HB	HBs	LH	LHs	LHsd	LHss	Mdsp	Mgp	Mhp	Marl	MR	MS	Msmud	MSs	WH	WHR
AL	0	0.18	0.1	0	0.12	0.14	0.09	0.15	0.12	0.09	0.07	0.19	0.12	0.08	0.1	0.09	0.06	0.09	0.04	0.03	0.03	0.15	0.06
Bgp	5990	0	0.13	0.18	0.14	0.17	0.11	0.11	0.15	0.12	0.12	0.22	0.2	0.13	0.17	0.12	0.12	0.15	0.15	0.17	0.17	0.23	0.2
BFi	1040	6080	0	0.07	0.12	0.12	0.1	0.12	0.14	0.03	0.07	0.11	0.07	0.09	0.13	0.1	0.09	0.07	0.03	0.1	0.11	0.09	0.07
BFt	1050	6120	40	0	0.12	0.13	0.09	0.12	0.1	0.07	0.06	0.17	0.09	0.08	0.09	0.08	0.05	0.08	0.04	0.04	0.02	0.13	0.07
GHj	38035	34245	37290	37315	0	0	0.06	0.1	0.08	0.08	0.04	0.1	0.12	0.07	0.14	0.11	0.1	0.08	0.06	0.13	0.11	0.12	0.09
GH	37470	33695	36715	36740	565	0	0.07	0.09	0.07	0.08	0.07	0.11	0.14	0.07	0.13	0.12	0.11	0.1	0.08	0.14	0.12	0.13	0.12
Hbd	20615	16145	20055	20085	18315	17780	0	0.06	0.04	0.06	0.05	0.16	0.08	0.08	0.09	0.09	0.09	0.03	0.06	0.09	0.07	0.15	0.09
HB	20720	16220	20125	20160	18245	17710	80	0	0.02	0.07	0.01	0.14	0.08	0.09	0.12	0.12	0.12	0.06	0.09	0.12	0.1	0.13	0.12
HBs	20680	16195	20080	20100	18250	17715	95	100	0	0.08	0.04	0.17	0.12	0.09	0.09	0.12	0.12	0.05	0.09	0.11	0.09	0.17	0.11
LH	40925	35040	41110	41155	37885	37675	30240	30220	30325	0	0.02	0.12	0.07	0.05	0.12	0.08	0.08	0.04	0.05	0.07	0.07	0.09	0.1
LHs	40450	34575	40650	40690	38030	37815	30060	30040	30140	680	0	0.12	0.07	0.04	0.11	0.06	0.04	0.06	0.07	0.03	0.04	0.1	0.11
LHsd	41015	35130	41205	41245	38070	37860	30400	30380	30480	180	650	0	0.23	0.07	0.12	0.12	0.1	0.15	0.12	0.15	0.14	0.12	0.18
LHss	40700	34815	40890	40935	38045	37830	30205	30185	30285	410	290	370	0	0.11	0.2	0.07	0.1	0.1	0.11	0.12	0.09	0.13	0.14
Mdsp	32280	26420	32490	32530	34645	34330	23490	23480	23560	8675	8180	8760	8425	0	0.06	0.04	0.04	0.08	0.06	0.07	0.05	0.08	0.11
Mgp	32815	26960	33015	33060	35460	35145	24310	24300	24400	8275	7735	8340	8000	845	0	0.09	0.08	0.13	0.1	0.09	0.06	0.15	0.14
Mhp	32665	26820	32885	32930	35065	34755	23980	23970	24070	8330	7810	8400	8065	495	390	0	0.01	0.06	0.06	0.12	0.06	0.12	0.1
Marl	32365	26520	32580	32620	34910	34590	23715	23708	23800	8625	8110	8695	8365	265	600	305	0	0.07	0.04	0.09	0.05	0.12	0.07
MR	31865	26035	32090	32130	35045	34720	23550	23540	23640	9170	8640	9240	8900	720	930	855	605	0	0.08	0.07	0.09	0.15	0.12
MS	670	6120	390	390	37615	37050	20420	20345	20375	41135	40665	41230	40915	32500	33025	32895	32590	32100	0	0.04	0.06	0.1	0.04
Msmud	220	6025	825	825	37890	37330	20620	20545	20575	40995	40520	41085	40780	32350	32860	32740	32435	31940	445	0	0.03	0.11	0.08
MSs	160	5960	895	895	37895	37340	20600	20525	20560	40915	40040	41005	40685	32270	32785	32665	32360	31860	525	100	0	0.12	0.09
WH	11365	5935	11715	11760	34750	34220	16520	16580	16600	29695	29200	29775	29450	21025	21495	21395	21095	20575	11460	11670	11375	0	0.15
WHR	11070	5610	11405	11450	34650	34120	16400	16465	16480	29960	29465	30045	29470	21290	21770	21665	21365	20850	11165	11365	11075	330	0

Table 23: Results of Mantel Tests to compare the genetic distance between populations. *Z*: Mantel test statistic, *r*: correlation coefficient.

Results of Mantel Tests implemented in IBD software			
	Z	*r*	*P* value
Comparison between sites	2241517.4	-0.001	0.52
Comparison within sites	13142.1	r=0.908	< 0.001
Comparison between all populations	10166527.1	r=0.798	< 0.001

The Mantel Tests show highly significant results for isolation by distance within sites. Here it was expected that gene flow can take place between ponds, and the IBD results show that this has indeed been the case (Table 22). This association completely disappears when considering the between-site comparisons. In these cases, ponds are too far away from each other to be connected through migration, and so the effects of habitat fragmentation and historical drift predominate. The comparison between all populations is highly significant because it includes the populations which are on the same sites. However, *r* is smaller than in the comparison within sites.

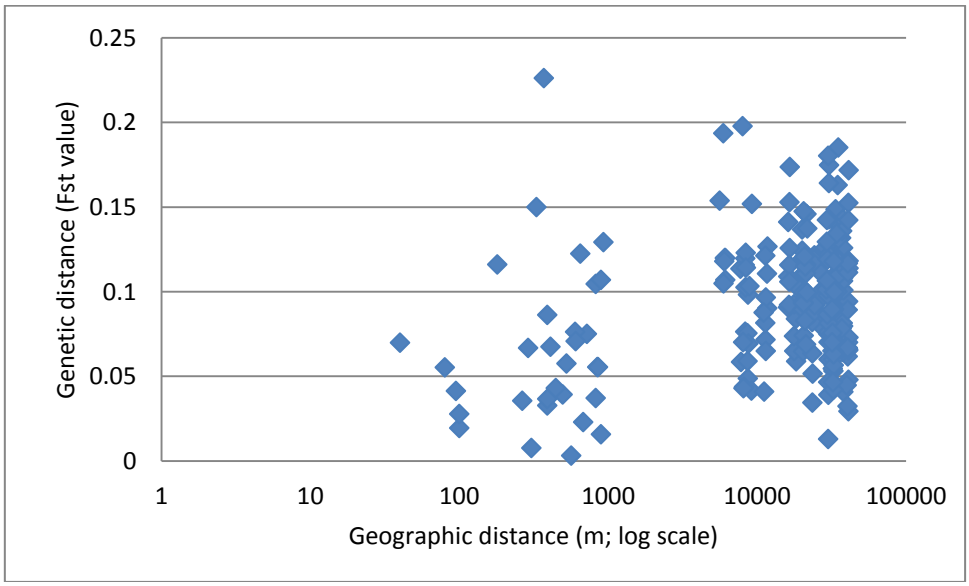


Figure 33: Overall isolation by distance.Each point represents a pairwise comparison between all possible combinations of ponds.Therefore there are 210 points shown on this graph for the 21 populations. Those populations which are within 1000m of

each other generally show low F_{ST} values, whereas those over 10 000m apart show higher F_{ST} values indicating a higher degree of differentiation.

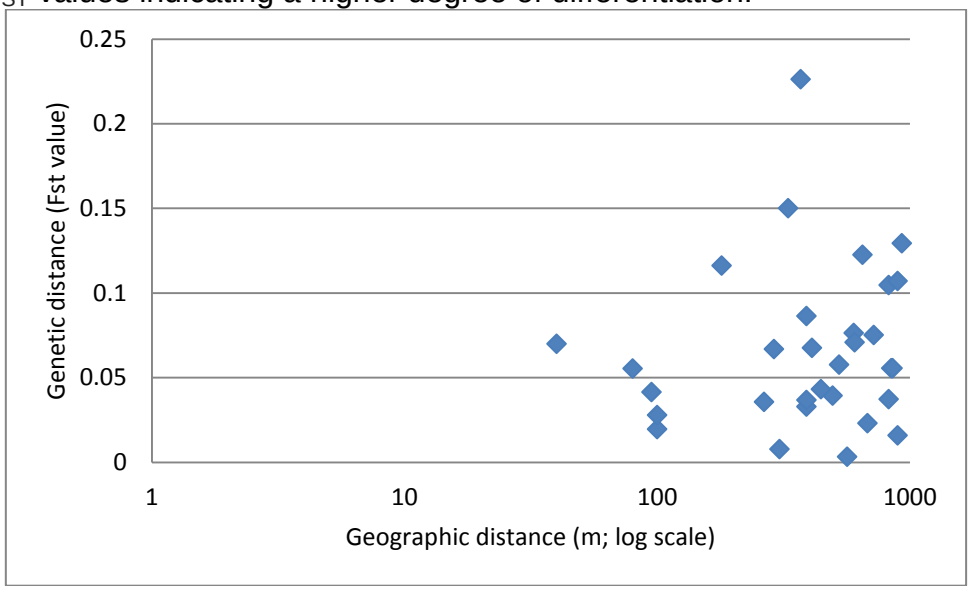


Figure 34: Isolation by distance within sites. This shows that individuals can disperse between ponds within sites where there is a significant isolation-by-distance pattern.

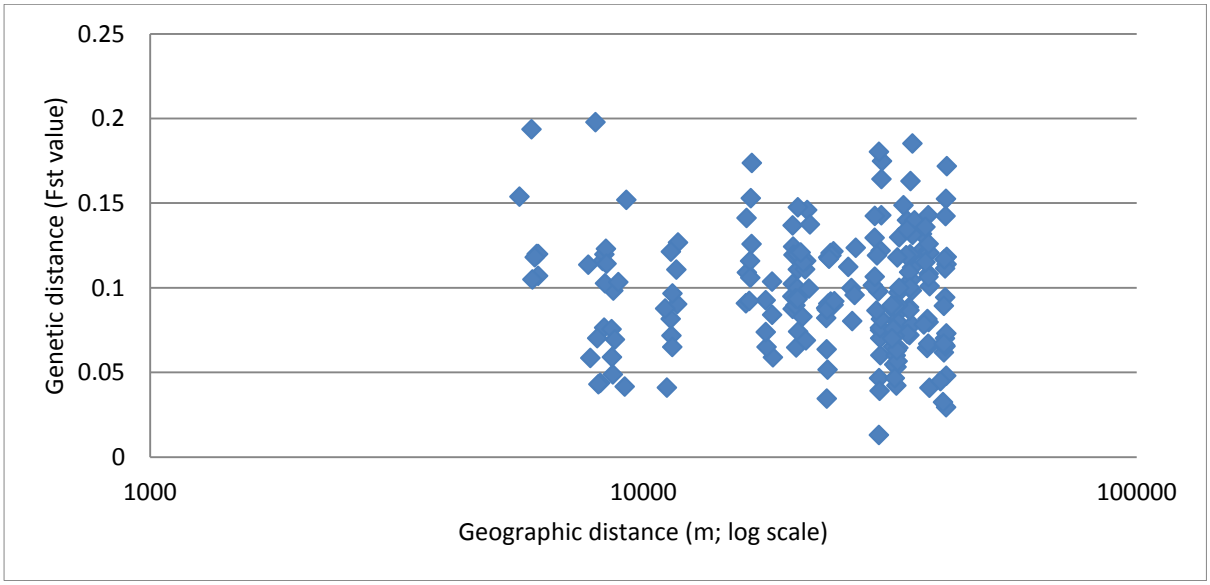


Figure 35: Isolation by distance between sites. This shows that individuals cannot disperse between sites where there is not a significant isolation-by-distance pattern.

The results of the BAPS analysis, where populations were assigned to the same cluster on the basis of similarity of alleles, are shown inTable 24. BAPS reduced the 23 populations from seven sites into nine genetic clusters, each represented by a different colour. The geographic relationship of the BAPS clusters is shown in Figure

36. All ponds at HB and GH are represented as their own discrete cluster (pink and yellow respectively). Four out of five ponds at Marl and four out of six ponds at MS are represented as green and red clusters, respectively, which suggests that genetic mixing is occurring between these ponds. Two out of four ponds at LH were shown as the same cluster but the two WH ponds were shown as different genetic units. The clusters at LH, Marl and MS are shown at a larger scale in Figures 37-39.

Table 24: Population clusters identified by BAPS. Each colour represents a total of 9 different clusters, to which populations have been assigned on the basis of similarity of alleles.

BAPS cluster	Pond	Site
	AL	Moss Shaw
	BFt	Moss Shaw
	MS	Moss Shaw
	Msmud	Moss Shaw
	MSs	Moss Shaw
	BFi	Moss Shaw
	Bgp	Bolton garden pond
	GHj	Gorse Hill
	GHm	Gorse Hill
	HBd	Hic Bibi
	HBm	Hic Bibi
	HBs	Hic Bibi
	LHm	Lane Head
	LHs	Lane Head
	LHsd	Lane Head
	LHss	Lane Head
	Mdsp	Marlings
	Mgp	Marlings
	Mhp	Marlings
	Mmp	Marlings
	MR	Marlings
	WH	Wittlestone Head
	WHR	Wittlestone Head

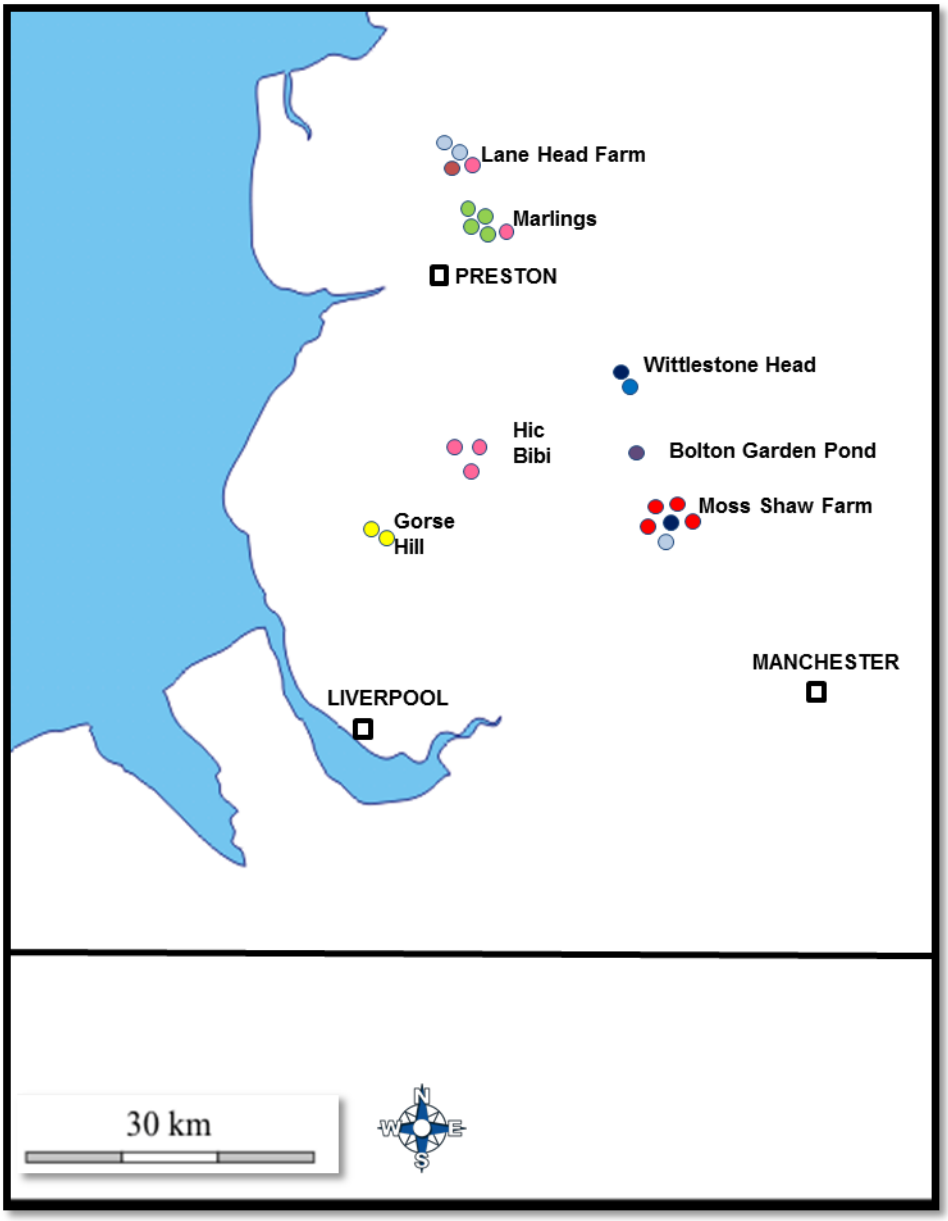


Figure 36: Population clusters identified by BAPS. The colours represent populations identified as genetically similar.

Figure 37: Four populations at Lane Head were assigned to three different clusters by BAPS.

Figure 38: Four populations (shown in green) at Marlings were assigned to the same cluster by BAPS. The fifth (shown in pink), over 600m away, was assigned separately.

Figure 39: Four populations at Moss Shaw Farm (shown in red) were assigned to the same cluster by BAPS. Two, including the largest population (shown in dark blue), were assigned separately.

5.3.2 Does isolation have any measurable or adverse genetic effects on *T. cristatus* populations?

The more isolated ponds in this study are not markedly poorer in genetic variation. The level of observed heterozygosity at GH was the 12th highest out of 23 populations in the study and at GHj it was 5th. GH had the 9th highest number of mean alleles per locus and GHj was 11th, The other isolated pond, Bgp, had one of the lowest levels of genetic variation but this is most likely related to its small population size. The peak estimated population supported by this pond was 42 +/-5.9 whereas at GH it was 753.0 +/-29.2.

5.3.3 Investigation to ascertain the success of a *T. cristatus* introduction.

The success, from a genetic standpoint, of the introduced population at Bgp was assessed on the basis of observed heterozygosity and mean number of alleles per locus, Bgp had the smallest level of observed heterozygosity and one of the smallest number of alleles per locus relative to other populations in this study. However, these levels were comparable with other small populations included in the study. There were no marked genetic effects following the introductions.

5.4 Discussion

Allele frequencies give measures of expected (H_e) and observed (H_o) heterozygosity in a population. An excess in homozygotes could result from inbreeding, whereas excessive heterozygotes may indicate either an influx of new individuals or a loss of homozygotes due to inbreeding depression. Loss of alleles and an increase in homozygotes could also be due to genetic drift, which is stronger in small populations. High genetic drift is also common following population bottlenecks (e.g. Jehle & Arntzen, 2002).

5.4.1 Methodological considerations

As expected, it was easier to collect eggs from some ponds than others. Finding eggs in ponds with the largest populations (GH and WH) was straightforward. Eggs were also easy to find at ponds such as LH and Mhp, even though neither population proved to be particularly large. At other ponds, where populations were likely to be small such as Bgp, Mdsp and MSmud, it proved very difficult to find eggs. At such ponds there was a high probability that a number of eggs from the same female were collected and this is recognised as a potential source of bias (but see Waples & Anderson, 2017). No eggs could be found at SF despite intensive searching in 2014 and 2015. However, more juvenile *T. cristatus* were captured here than at any other pond, demonstrating that successful breeding was taking place.

Although all PCR products were verified visually by agarose gel electrophoresis, some could not be size scored. Primer 29 showed a particularly high failure rate, and primer 13 and 50 were often difficult to size score with certainty. Low rates of errors during the size scoring process were recognised as a source of inaccuracy, with the potential to impact on the quality of the final dataset. It is useful to review the results of this study in the context of genetic data collected at other sites (Table 25).

Table 25: Descriptive population genetic data for *T. cristatus* found in 5 other studies.

Pond	mean number of alleles/locus	He	Ho	Loci out of HWE	Reference
Populations from Leicestershire, England					O' Brien et al., 2015
PF	3.67	0.43	0.53		
P	2.83	0.56	0.53		
CC	8.17	0.84	0.8	36	
G	8.83	0.82	0.8		
Populations from Scottish Highlands				N/A	O' Brien et al., 2015
BW	2.67	0.26	0.25		
CL	3.33	0.4	0.39		
D	2.67	0.44	0.37	27	
FGC	1.6	0.35	0.41		
MO	3.67	0.38	0.3	29	
NS	3.33	0.52	0.36	35	
PH	2.5	0.33	0.28		
LV	3.17	0.42	0.37	36	
Populations from Western France					Jehle et al., 2005
2A1		0.61	0.59		
2C8		0.57	0.53		
2E4		0.60	0.58		
2H6		0.63	0.67		
2N8		0.60	0.54		
2P7		0.62	0.64		
232		0.60	0.58		
233		0.49	0.49		
N3		0.55	0.52		
N6		0.62	0.55		
N7		0.59	0.57		
N8		0.55	0.50		
N10		0.62	0.51	35	
N11		0.65	0.53	13,29	
N13		0.56	0.52	35,43	
Flanders, Belgium					Schön et al., 2011
Tommelen	5.86	.65	.67	-	
Ieper	6.57	.64	.61	-	
Steendorp	5.14	.57	.59	-	
Oosthoek	3.86	.53	.54	-	
Westhoek	5.57	.62	.66	-	
Populations from Bavaria, Germany					Maletzky et al., 2010
Nie	2.85	0.48	0.48	-	
Sil	2	0.34	0.37	-	
Sur	3.62	0.59	0.49	-	
Populations from Salzburg, Austria				-	Maletzky et al., 2010
Irl	2.91	0.58	0.49	-	

Table 25 (continued)

Pond	mean number of alleles/locus	He	Ho	Loci out of HWE	Reference
Bue	3.4	0.59	0.55		Maletzky et al., 2010
Fue	2.95	0.55	0.58		
South-east Norway					Redford, 2010
Lille Mortetjern	4.75	0.6	0.64	46	
Skillebekk Dammen	5.75	0.7	0.63	27,29,32,35,43,46	
Hovindammen	3.5	0.5	0.45	27,36,46	
Ovre Skogsdam	6	0.6	0.65	29,36,43	
Branndammen	4.25	0.6	0.62	35,36	

Most populations are out of HWE at one or more loci. This is in contrast to other studies, for example Jehle et al., (2005) where only 3 out of 15 samples were out of HWE (Table 24), and could be due to many of the samples collected from ponds with small populations, or small sample sizes. The unintentional collection of eggs from the same female could also have been a contributory factor. For example, the population with the highest deviation was GHj where only a small number of eggs could be found. The problems arising from inadvertently collecting genetic samples from siblings have been described by Waples & Anderson (2017). Siblings occur naturally in all populations at frequencies that are inversely related to effective population size (N_e) and their removal would risk erasing part of the evolutionary signal of small populations. They also state that excluding siblings from analysis reduces the sample size, which sets up an inevitable trade-off with respect to precision and statistical power required to analyse the data. Therefore although eggs collected from GHj are likely to have been siblings, they still have the potential to provide valuable information and should not be discounted.

Genotyping error may also have been a reason for the unusually high number of loci being out of HWE. However, deviations from HWE were largely unbiased with respect to loci, discounting the possibility that they were caused by e.g. locus-specific problems in PCR amplification.

Genetic variation can be measured by the mean number of alleles per locus and through observed heterozygosity. A comparison of both is shown in Table 22. Only 1 out of the five populations with the highest mean number of alleles also has a high *Ho.* Only 3 out of 5 sites with the lowest number of mean alleles are the same as those with the lowest levels of *Ho*. The reason behind the differences lies in the frequency distributions of alleles. If, for example, there are several rare alleles in a population (perhaps present in only 1-2 individuals), this raises the average number of alleles significantly, but only very marginally raises the heterozygosity because only a very small number of individuals are involved. Conversely, if allele frequencies in a population are very evenly distributed, then heterozygosity is high at moderate or even low levels of allelic diversity. The lowest levels of heterozygosity at Bgp and Mgp probably reflect small populations. In both cases several visits to the pond were made, eggs were easy to find and small numbers collected on each occasion. The samples collected should have been representative of the population, and the low number of alleles per locus is probably an accurate representation of their low population sizes. It was expected that the introduced population of Bgp will have a relatively high level of genetic variation since it was founded by releasing individuals from two different sites. However, it had the lowest observed heterozygosity, and one of the lowest numbers of alleles per locus. This could reflect the genetic similarities between populations in South Lancashire in that mixing individuals from two ponds has not significantly increased the genetic variation of the resulting population. It could also show that, even if an increased level of genetic variation was achieved immediately after the introduction, this may already have been lost due to genetic drift given the small number of individuals. Genetic drift can lead to the loss of alleles from small populations, and in isolated ponds cannot be compensated for by new alleles arriving through immigration. If this was the case, small populations may exhibit a reduction in genetic diversity after a relatively short time, although samples from the founder population would be required to confirm this hypothesis.

The amount of genetic variation found in this study, even among isolated populations, compares favourably with values found elsewhere. H_o is higher (0.64 – 0.89) than those for populations at the edge of their range in the Highlands of

Scotland (H_o = 0.26 – 0.52) and higher than samples from six ponds in North West Austria (H_o = 0.37 – 0.58) where geographical isolation of populations was very high (Maletzky, 2010). They are also higher than at 15 ponds in western France (H_o = 0.49 – 0.67, Jehle et al., 2005), which can be explained by a recent expansion of the local range of *T. cristatus* in the study area into ponds which were not occupied by the species several decades ago. The H_o values found in this study are comparable with those found in their continuous UK range in Leicestershire (H_o = 0.53 – 0.80). For the vast majority of populations in this study, H_o is higher than H_e. Such results can be found in other studies (for example Schön et al., 2011) where *Ho* was higher than *He* in 6 out of 10 sites, but most other studies show the reverse (for example Jehle et al., 2005, Maletzky, 2010 and Jehle, 2013). While size scoring error cannot be ruled out, inbreeding depression and heterozygote advantage can account for this finding.

Figure 35 shows that populations within 1000 m of each other generally have low F_{ST} values, whereas those over 10 000 m apart show higher F_{ST} values indicating a higher degree of differentiation. This demonstrates that populations on the same site (within 1000 m of each other) have undergone genetic mixing with adjacent populations therefore migration of some individuals between adjacent populations must have taken place. Similarities among populations over 10 000 m apart, shown by the clustering of points between F_{ST} values of 0.05 and 0.15 on Figure 35, demonstrate that all populations are relatively closely related. Only one comparison has an F_{ST} value greater than 0.2. This geographic similarity is most likely due to the residual effect of post glacial colonisation. It shows that even populations separated by much greater than the maximum dispersal distances for *T. cristatus* share many genetic similarities.

Measures of genetic differentiation (F_{ST}) were relatively low when compared to other studies (for example Jehle et al., 2005 where F_{ST} = 0.05 – 0.52 and Maletzky, 2010 where F_{ST} = 0.18 – 0.52). The amount of genetic differentiation was generally higher than ponds in central England, but markedly lower than ponds in the Highlands of Scotland where the species is at the edge of its range (Jehle et al., 2013). The North West of England is located approximately mid-way between these two regions, corresponding to intermediate F_{ST} values. Low levels of genetic differentiation could

be due to a historic high density of ponds and waterbodies in this region, facilitating high gene flow between populations in the past.

5.4.2 Investigation into the dispersal of individuals between ponds in the modern agricultural landscape.

The results provided by IBD show a significant relation between genetic distance and increasing geographic distance within sites as well as across the entire study area. This confirms that gene flow is still possible at least between nearby populations.

The BAPS analysis confirms that many of the ponds which are close to each other are inhabited by populations which are genetically similar. This conforms to the expectation of migration of individuals between ponds and the resulting genetic mixing, and fits with other studies on dispersal abilities of amphibians (Smith & Green, 2005; Jehle & Sinsch 2007, Sinsch, 2014). C-M-R studies have shown that most crested newts overwintered close (less than 100 m) to their spawning sites but dispersal distances of between 500 and 1.6 km have been observed (Stoefer & Schneeweiß, 2001; Haubrock & Altrichter, 2016). Dispersal distances of 860 m for juveniles have also been recorded (Kupfer, 1998). Populations which are clustered together at Marl and MS are well within these distances.

At the non farmed sites of GH and HB, dispersal between ponds appears to be facilitated by dense unmanaged vegetation and woodland. All populations at both sites were allocated to their actual geographic clusters by BAPS, confirming that genetic mixing between ponds had taken place. At Hic Bibi this could be expected as the ponds are close together (within 100m) and the terrestrial habitat is good which facilitates dispersal. Whether habitat quality at the farm sites was of sufficient quality to allow dispersal between ponds was one of the key questions for the genetic study. The results clearly show that genetic mixing has occurred between populations at Marl and MS, but the results are less clear in the case of LH as BAPs characterised populations at the four adjacent ponds as being from three distinct populations.

Three ponds were clustered with others from different sites (MR, LHs and BFt, Figures 38-41) and a further pond at Lane Head Farm, LHsd, was allocated to a cluster of its own. In the case of MR, this is likely to be a sample size issue, as only 8 eggs were genotyped. It was also the most geographically isolated pond, being almost 600 m from the nearest occupied pond. Genetic drift could therefore help to explain why this pond is differentiated from its neighbours. At LHs, LHsd and BFi, collection of eggs from the same female could have been a source of bias. At these ponds eggs were collected during one visit only. At LHsd, a maximum of one individual was captured during fieldwork so it is likely that this population was very small. This would have resulted in high levels of drift leading BAPS to interpret it as a distinct population. It is also worth considering that BAPS is sensitive to genotyping error.

5.4.3 Does isolation have any measurable or adverse genetic effects on *T. cristatus* populations?

Using the methods employed by this study, the populations at GH showed no adverse effects of isolation as they exhibited both a relatively high observed heterozygosity and high mean number of alleles per locus. The isolated population at Bgp showed a much lower level of genetic variation which was probably due to two factors, (i) it was founded with 40 individuals which could be seen as a population bottleneck and (ii) the population remained relatively low, reaching a recorded peak of 42.0 +/- 5.9 in 2013 and falling to just 15 +/-2.2 in 2015. Both factors would limit the genetic diversity of this population and increase the loss of genetic diversity through genetic drift. It is important to note that the introduced population used individuals from two different founder populations from the Greater Manchester area. This should have increased the level of diversity in the introduced population but this is not reflected in the data from this study. There are two possible explanations for this: (i) that the original population did have a relatively high level of genetic diversity which has been lost over the last 24-25 years or (ii) that the original populations were relatively similar, Since there is no genetic data available from the original introduction, neither of these explanations can be disproved.

5.4.4 Investigation to ascertain the success of a *T. cristatus* introduction.

Bgp, the introduced population, had a small number of alleles per locus relative to other populations in this study, which may have been predictable bearing in mind that it was founded with 40 individuals. Nevertheless it had more alleles per locus than AL, LHss, LHsd, Mgp, MR, all farm ponds identified as small populations. It also had more alleles per locus than all of the *T, cristatus* populations in the Scottish Highlands (O'Brien et al., 2016) and more than populations in two studies from Germany and Austria (Maletzky et al., 2010). This demonstrates that although the level of genetic variation at Bgp was relatively low, it was above the level at which natural populations are able to thrive. Thus this introduction can be regarded as a success from a genetic standpoint.

Chapter 6: General discussion

The overall aim of this thesis was to investigate *T. cristatus* populations on farmland to find out whether aspects of their population structure or demography gave cause for conservation concern. In order to do so it compared populations on farmed sites with those on non farmed sites. It covered three areas of research: population size, population age structure and population genetic structure. The results demonstrated that farmland populations showed no inherent problems compared to those on non farmed sites. Consequently the habitat value of farmland for this highly protected European Protected Species should not be underestimated.

6.1 Population size

The investigation into population size was conducted to ascertain the scale of farmland populations which could then be compared with those on non-farmed sites. The estimates were calculated using the Begon Weighted Mean which relied on the assumption that each population was closed. The alternative of using an open population model to calculate population size was inhibited by a lack of data on capture probability and an ability to distinguish between mortality and migration.The assumptions required for the Begon Weighted Mean is recognised as a source of potential error as it is likely that some individuals entered or left the pond during the capture period. However, since the source of error is consistent between populations the results provide valid comparisions. The programme MARK was used to estimate the size of the garen pond population (Figure 12) and the result was similar to that calculated with the Begon Weighted Mean. This indicated that it was an appropriate method to use throughout this study.

Population estimates showed that even where pond density and the level of pond occupancy was high, *T. cristatus*, populations in farmland can be very low. The five Moss Shaw ponds were occupied by *T. cristatus* but four provided a very small number of captures. Only one provided sufficient recaptures to estimate population size, which was estimated at 225 and 245 in 2014 and 2015, respectively. Three of the small populations were in small water bodies (under 130m^2), one of which dried

out completely in mid-summer. The two Marlings ponds supported relatively large populations, three supported smaller populations which could not be estimated due to a lack of captures or recaptures and one pond was unoccupied. Population estimates were conducted for two small populations at Lane Head Farm and were calculated as 7.8 +/-6.8 and 5.8 +/-5.1. Nevertheless, each farm included one population which was substantially greater the surrounding ponds and these varied from 51.1 +/-5.6 at Lane Head, to 331.4 +/-65.7 and 417.2 +/-159.4, at Marlings and Wittlestone Head respectively. Population survival probably depends on these source ponds. The largest population recorded in this study was 753.0 +/-29.2 at Gorse Hill, an isolated, non farmed site. Good aquatic and terrestrial habitat quality is likely to be the reason for this large population size but further research to establish the relationship between habitat quality and population size would be valuable. For example it is reasonable to assume that populations on farmland are always likely to be smaller than those on sites with better habitat quality but it would be possible to confirm this by quantifying habitat quality. An attempt was made to compare aquatic habitats using PSYM but it was not possible to do this adequately within the scope of this thesis.

The population studies illustrate the key importance that a single pond can have at a particular site. Some landowners may be amenable to managing one pond for *T. cristatus* but may not wish to manage several ponds. When resources are limited, it is helpful to understand where they can be used to best effect. It is therefore important that the source pond is identified, and given priority for appropriate management. This could include a buffer strip around the pond edge, removal of trees to reduce shading and in ponds heavily impacted by cattle a fence could be erected to allow marginal vegetation to grow. Providing areas of unmanaged vegetation, especially surrounding a pond, are likely to be particularly beneficial to juveniles since within the first few months or years of life, they utilise terrestrial habitat close to natal ponds on a semi-permanent basis and occupy a small home range (Jarvis, 2012). As pond density is the habitat factor which most positively influences *T. cristatus* presence (Brady, 2017), and the creation of new ponds is therefore always likely to be beneficial for *T. cristatus*.

6.2 Fluctuations in population size

Estimated population size generally fluctuated between years and the reasons for this varied. Changes at Lane Head indicated a consistent decline and the relatively small changes at the garden pond were likely to be part of cyclical population changes documented elsewhere (Jehle et al., 2011). Some populations estimates showed marked changes between years, for example at Wittlestone Head the variations were between 417 +/- 159.4 in 2014, 193 +/- 47.0 in 2015 and 290 +/- 20.47 in 2016. There was no apparent reason for the huge decline in the estimated population between 2014 and 2015 and this study was unable to ascertain whether these changes were due to mortality or newts not returning to the pond to breed. Whether these fluctuations reflect genuine changes in population could be investigated by comparing the identity of individuals captured in different years. If a high proportion of newts captured in 2014 were not seen in 2015 but captured again in 2016, it could be assumed that they had not returned to the pond to breed in 2015. This is possible, as a high probability of *T. cristatus* missing breeding opportunities was found in a study conducted by Schwizer, 2007. Alternatively, if those newts from 2014 were not seen again in 2015 or 2016, the reduced estimate in 2015 would reflect a true population decline. This data could be used to give a measure of survival between years and could be compared with that for other sites.

Population estimates from a total of 6 ponds declined between 2014 and 2015 suggesting that the lower estimates in 2015 could be due to environmental factors. Temperature and rainfall may be responsible, as mild wet winters have been identified as adversely affecting both *Bufo bufo* and *Triturus cristatus* populations (Reading, C., 2007, Griffiths, et al., 2010,). This could be investigated by comparing the population estimates with climatic data.

The large increase in the population estimate for Marlings Hedge pond was most likely a result of changes in capture technique which illustrates the importance of consistency in fieldwork. The effect of such inconsistencies may be difficult to find and may not be apparent when reviewing data from other studies.

6.3 Survival and detectability

Data from the garden pond study showed that of the 55 different individuals identified, only one (a female) was captured in all five years and only eight were captured in four years of the study. The reasons for this can only be speculated, but this observation may reflect high levels of mortality. It is possible that the newts which returned in several consecutive years resided close to the pond during their terrestrial phase where the risk of mortality is likely to be low, whereas those which migrated furthest from the pond would be at greatest risk of mortality, for example by falling into gulley pots or being killed on roads. The study shows a high level of recruitment, for example in 2016, 7 of the 19 individuals captured were new recruits. This indicates that the risk of mortality in the terrestrial habitat is a key limiting factor for population size.

The garden pond study demonstrated that even with intensive capture effort and high detectability (calculated by MARK as 0.91) at least 8 capture visits over April and May are required to capture each individual at least once. Fewer visits may provide the basis for statistically accurate population estimates, such as at Lane Head in 2016 when it was possible to obtain a population estimate with a SE of only 5.6 after just 6 visits. In order to avoid unnecessary survey effort by conducting more capture visits than required to obtain a statistically accurate population estimate, the best approach would be to compare belly patterns and calculate the population estimate after each visit. As soon as the SE error becomes low (which needs to be proportionally smaller for small populations), capture visits can be discontinued. Fewer visits are likely to be needed where capture effort is high and for populations with high detectability.

6.4 Operational sex ratios

Sex ratios of 1:1 are reflected in some but not all of the population estimates calculated by this study (Table 7). However at three populations (the garden pond, Gorse Hill and Lane Head Farm) there was a consistent sex bias across the years. At the garden pond from 2013 onwards the number of females captured was more than double the number of males. The male and female population estimates followed the same pattern (Table 7). At Lane Head Farm population estimates for females were also markedly higher than those for males in three out of four years, but in the case of Gorse Hill the male population estimate outnumbered the female over three consecutive years. Such differences in sex ratios have been found in other studies (Jarvis, 2012). What causes such imbalances, whether they are temporary or long term phenomenon and what effect this has on population size is a topic for future research.

6.5 A comparison of age structure at farmed and non farmed sites

There was a high degree of variability between population age structures. No distinction could be drawn between those on farmed and those on non farmed sites. Despite there being no significant relationship between population size and age structure, there is some evidence to suggest that a large proportion of older individuals in a population is a sign of decline. This was the case at Lane Head Farm, where the population declined between2013 and 2016 and 18 individuals over the age of 9 were found. It was also likely to be the case at Acorn Bank, where 24 newts over the age of 9 were found. (In this case a large population was confined to a small pond and cannibalism of larvae is likely to be the reason why few young newts were present.) This contrasts with the highest population estimate in this study, at Gorse Hill, in which only one adult over 9 years old was found. The population at Rixton Claypits was probably much larger than any estimated in this study and here no individuals over 9 years old were found. The reasons for this are unknown and merit further research. However, this observation supports the suggestion that older individuals, which have a lower BCI than younger ones, may return early to the pond in order to compete with younger newts. If older newts are

less able to compete effectively for resources their ability to compete in populations of high density would be further reduced. This would explain why few old newts were found at Gorse Hill, but what happens to the older newts in these situations is unknown. Given that they are capable of surviving to a maximum of 15 years (in this study) it could be that in these situations they do not attempt to compete during the breeding season and either remain entirely terrestrial, or attempt to find new ponds. This could explain why a very large adult female (85 mm and 16.8 g) was found in a newly created pond at Lane Head Farm in 2014.

The median estimated age across all populations was seven years. Few very young or very old individuals found in most populations. The most convincing explanation as to why there is not a preponderance of young individuals appears to be that sexually mature adults do not return to breed for a number of years. The reason why sexually mature adults are not taking part in breeding activity, and how and where these individuals spend their time, is also worthy of further investigation.

6.6 Comparison of body condition at the farmed and non farmed sites

Similar to age structure, BCI and size also varied markedly between populations and sexes. For example, females captured at LH were large (62.61% were over 80mm), and median female BCI was the highest of all populations. However, only 15.38% (n=78) of males were over 80mm, which had a median BCI ranked only 10th among 14 populations. BCI was unrelated to whether populations inhabited farmed or non-farmed sites.

It would have been reasonable to expect that newts in the more structurally varied habitats, primarily those at GH and to an extent WH, would have a higher BCI than those at farm sites with little structural diversity, such as MS. However this was not the case and in fact WH was the only site with negative BCI values for both males and females, with BCI of males and females being significantly different from those in nine and eight other populations, respectively. This study confirms the findings of an earlier study (Hagstrom, 1980) that it was not possible to assume that small individuals are young. In this thesis, data in general terms show that short individuals are often younger than long ones. For example, 68 individuals measured at 60-

65mm SVL had an estimated age of between three and nine years, with the majority being between four and six years of age. 102 individuals measured at 80-92mm SVL varied between 5 and 15 years of age, with the majority being between 9 and 11 years old.

6.7 Investigation into the dispersal of individuals between ponds in the modern agricultural landscape.

Whether habitat quality at the farm sites was of sufficient quality to allow dispersal between ponds was one of the key questions for the genetic study. The results show that genetic mixing has occurred between adjacent populations in the farmed landscape therefore gene flow is still possible at least between nearby populations.

The results provided by IBD show a significant relation between genetic distance and increasing geographic distance within sites as well as across the entire study area. The BAPS analysis confirms that many of the ponds close to each other are inhabited by populations which are genetically similar. Genetic mixing occurred between populations at Marl and MS, but the results are less clear in the case of LH. The BAPS results and the inferences based on F_{ST} indicate that the dispersal of individuals between farm ponds is taking place, and that these populations are functioning as metapopulations.

The period over which agricultural changes have led to a decline in the quality of the farmed landscape for *T cristatus* is short within the context of evolutionary history, maybe representing around ten to fifteen generations of the species. Therefore it is possible that the timescale of these changes is too short for any genetic effects to be seen. It is also possible that farmland habitats included in this study have not fundamentally changed over the last 70 years or so, making it more difficult to identify any genetic effects of changing farming practice. It is possible that different results would have been obtained if populations separated by intensively managed

arable habitats had been included in this study. This was not possible as part of this thesis as no such sites could be identified prior to the start of fieldwork in 2013. Nevertheless, this study confirms the ability of *T. cristatus* to disperse across modern farmland. It also provides a valuable dataset which can be used for comparison with future studies.

6.8 Does isolation have any measurable or adverse genetic effects on *T. cristatus* populations?

This study found no adverse effects of isolation on *T. cristatus* populations therefore genetic factors are not an obstacle to the conservation of *T. cristatus*. Thus where populations are in decline, environmental factors are likely to be the main contributory factors. The isolated populations at GH both exhibited a relatively high observed heterozygosity and high mean number of alleles per locus. The isolated population at Bgp showed a much lower level of genetic variation which was probably due to two factors, (i) it was founded with 40 individuals which could be seen as a population bottleneck and (ii) the population remained relatively low, reaching a recorded peak of 42.0 +/- 5.9 in 2013 and falling to just 15 +/-2.2 in 2015. Both factors would limit the genetic diversity of this population and increase the loss of genetic diversity through genetic drift.

These results are positive for the conservation of *T. cristatus* as there are probably a large number of isolated populations scattered across the farmed landscape. These results show that genetic factors are very unlikely to contribute further to their decline. However, as found by this research although such populations can exist at very low levels, their risk of extinction is increased by isolation and small populations on isolated sites will remain at particular risk of extinction due to stochastic factors. Even large isolated populations are predicted to be at relatively high risk of extinction over a fifty year period (Griffiths & Williams, 2000). Therefore although the genetic structure of *T. cristatus* populations can withstand the pressure of isolation, isolation remains an important factor which adversely affects chances of long term survival. Habitat fragmentation remains a threat to *T. cristatus* and the results of this research do not diminish this.

6. 9 Investigation to ascertain the success of a *T. cristatus* introduction

Bgp, the introduced population, had a small number of alleles per locus relative to other populations in this study which may have been predictable bearing in mind that it was founded with 40 individuals. Nevertheless it had more alleles per locus than AL, LHss, LHsd, Mgp, MR, all farm ponds identified as small populations. Although the level of genetic variation at Bgp was relatively low, it was above the level at which natural populations are able to thrive therefore it can be regarded as a success from a genetic standpoint. This population was founded by individuals from two different populations but whether the current level of diversity represents a loss over the last 24-25 years cannot be ascertained due to an absence of data from the founder population. A future follow-up study would be able to confirm whether genetic diversity in a small isolated population is lost over time, or whether it remains relatively constant.

6.10 The future of Triturus cristatus conservation in the UK

The next five years will be a time of great change for *T. cristatus* conservation in the UK. Legal protection for the species under the Habitats Regulations and Wildlife and Countryside Act has been the incentive for much of the conservation work conducted. Given that the UK is set to leave the European Union in 2019, this legal protection is by no means assured. An article in the Financial Times (Parker, 2017) reported that “Government figures have told the Financial Times that the EU Habitats Directive is among measures set to be repealed, citing the “excessive” protection given to the amphibian (i.e. great crested newt) as a reason to change the law.” Reduced protection for *T. cristatus* in the UK appears likely when considered against the backdrop of relentless bad publicity for the species. Articles critical of *T. cristatus* have seen a huge increase since 2000 (Perkins, 2014). This has included national newspaper headlines such as “Builder forced to spend £1m to relocate 150 newts” (Daily Mail, 2014) and a leading story in the Sun “£1.7m newtance” reporting that a single newt may have cost the British taxpayer £1.7million (The Sun, 2008).

A reduced level of protection for the species would affect conservation efforts in a number of ways. The proactive conservation of *T. cristatus* in England has often been left to charities and voluntary bodies (Gent, 2001), and funding has often been dependent on the protected status of the species. From 1995 to 2012, a key focus of activity was the Great Crested Newt Species Action Plan, encompassing national and local tiers. Unfortunately, communication between the tiers was poor and there was little coordination to ensure they worked together effectively. The absence of a clear statutory basis for the plans often meant that they were not taken seriously by many local authorities, who did little to take the plans forward (Gent, 2001). Nothing has replaced the Species Action Plan process so there is now an absence of an inclusive national forum for developing initiatives for *T. cristatus* conservation. In the current economic climate, financial support for any conservation project is difficult to obtain, and funding for widespread amphibians is particularly problematic as they are not perceived as being at risk of extinction. Without their priority status, attracting funding for *T. cristatus* conservation projects will be extremely challenging.

Changes in the legal status of *T. cristatus* could have a profound effect on mitigation work to compensate for the effects of development. Whilst this issue is outside the scope of this thesis, it is worth considering briefly since development is a main threat to the habitat of *T. cristatus*. The economic downturn since 2008 has focussed attention both on the cost implications of legal protection for the species and associated delays to the planning process. In relation to British transport policy Aldred & Tepe (2011) noted that “enthusiasm for the environment had waned as an age of austerity had cast environmental protections as an unaffordable luxury”. It is impossible to quantify the amount of money spent by developers on *T. cristatus* mitigation projects, but their total cost in 2010 was estimated to be between £60 – 125 million (Lewis et al., 2012). More recently, Natural England (NE) has estimated the cost implications of EPS Licensing for *T. cristatus* to be approximately £45 million per year (Cameron, HWM, 2017). Fundamental changes to the licensing system are already underway, with responsibility for licences being passed from NE to Local Authorities. New District Licences will permit the destruction of *T. cristatus* habitats (with the inevitable killing of individuals which has hitherto been illegal) as long as developers pay a fixed sum to improve habitats for the species elsewhere (Woking

Borough Council 2016). This approach has been promoted by NE as a pragmatic approach to conservation of *T. cristatus*, but whether this will be successful in maintaining favourable conservation status for the species remains to be seen. As discussed in the Introduction, there have been few benefits for *T. cristatus* from agri-environment schemes so the impact of a change in legislation upon on such schemes would be negligible.

Although a reduced level of protection for *T. cristatus* is likely to lead to adverse impacts, there may also be some benefits. Farmers, landowners and their land agents are generally aware of the protected status of the species and consequently there is a widespread perception that its presence has negative consequences for a land manager. This perception is hard to dispel, and it is understandable that conservation measures for the species can run into opposition from farmers and landowners. There is much anecdotal evidence that landowners link the presence of *T. cristatus* to unnecessary bureaucracy and interference from government agencies. Ensuring that *T. cristatus* ponds remain in good condition cannot be done by legal protection alone. As the number of countryside ponds has been estimated at approximately 500 000 (Williams et al., 2008), the cooperation and goodwill of farmers and landowners is essential. Encouraging and supporting land managers should therefore be a high conservation priority and a partnership approach between them and conservation organisations must be the best way forward. A relaxation in the legislation and the licensing system could help allay fears arising from the presence of *T. cristatus*, making them more amenable to implementing conservation measures that could benefit the species.

Against this difficult backdrop, the need for more detailed knowledge about the ecology *T. cristatus* is as high as ever. This thesis has contributed to understanding of the ecology of the species in three key areas: population size, age structure and population structure. The results support recognised conservation objectives for *T. cristatus* of creating and managing ponds, and highlights the importance of the terrestrial habitat.

6.11 Summary of areas for further research

This thesis has produced a number of important results but it has also highlighted some key areas for further research. These can be summarised as the need to (i) quantify the effect of habitat quality on population size (ii) explain why sexually mature adults do not return to breed for a number of years and how they behave in the intervening period. (iii) conduct a similar study in a different part of the UK with very different landscape characteristics, in particular where populations are located within intensively managed arable land. The ability of *T. cristatus* to disperse in such landscapes is likely to be lower and due to intensive land management populations may be assumed to be lower. Whether this is the case is unkown but this study is unlikely to be representative of farmland populations across the UK.

6.12 Key conservation messages from this research

The findings of this research convey some positive messages for the conservation of *T. cristatus* in the agricultural landscape. There was no significant difference between the body condition of individuals on farm sites compared with non-farmed sites. Individuals in the farmed landscape survived to an estimated 14 years. This longevity indicates that both the aquatic and terrestrial habitat was sufficient to allow newts to fulfil their natural lifespan. The maximum estimate age for *T. cristatus* found in this study was 15 years, therefore individuals may be present in the landscape for a number of years after a pond is lost or otherwise becomes unsuitable. This means that any new ponds may be colonised by the remnants of a population which may have been assumed to be extinct.

Although the majority of individuals became sexually mature at 2-3 years, the results of this study indicate that a large proportion of adults may not return to the pond until they reach age 6-7. This means that the effects of any habitat management work may not be fully reflected in larger population sizes until 6-7 years after the work took place. This also means that until individuals reach 6-7 years of age, many are likely to spend this entire period of their lives in the terrestrial habitat, highlighting its importance.

This research into the genetic structure of populations has demonstrated that *T. cristatus* can disperse across modern farmed landscape and interact with adjacent populations. Thus, modern farming practices do not appear to have limited the ability of the species to disperse at the scale of study sites examined here. Fragmentation of habitat and isolation of *T. cristatus* ponds is an issue of conservation concern, yet this study showed that isolated populations showed no evidence of significant genetic deterioration. This indicates that given the availability of suitable habitat, isolated populations should be able to survive unimpeded by genetic constraints. Given the widespread distribution of *T. cristatus* across the UK countryside, the long-term conservation of the species must focus on these populations. This research should help focus attention on the need to do this.

Chapter 7: References

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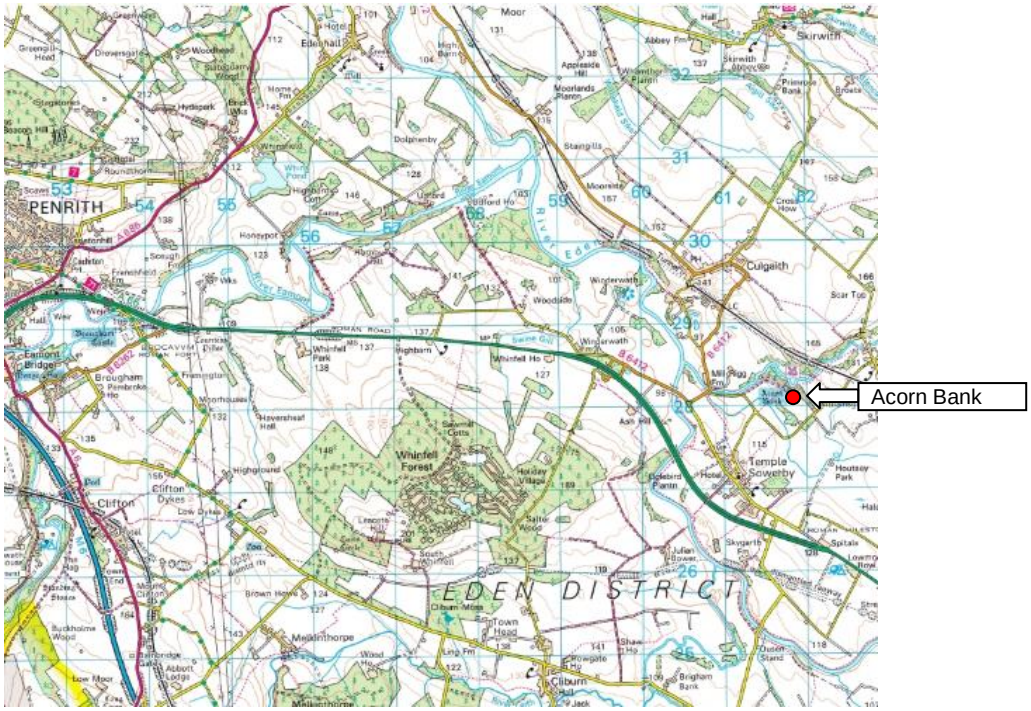
Appendix 1: Location of study ponds

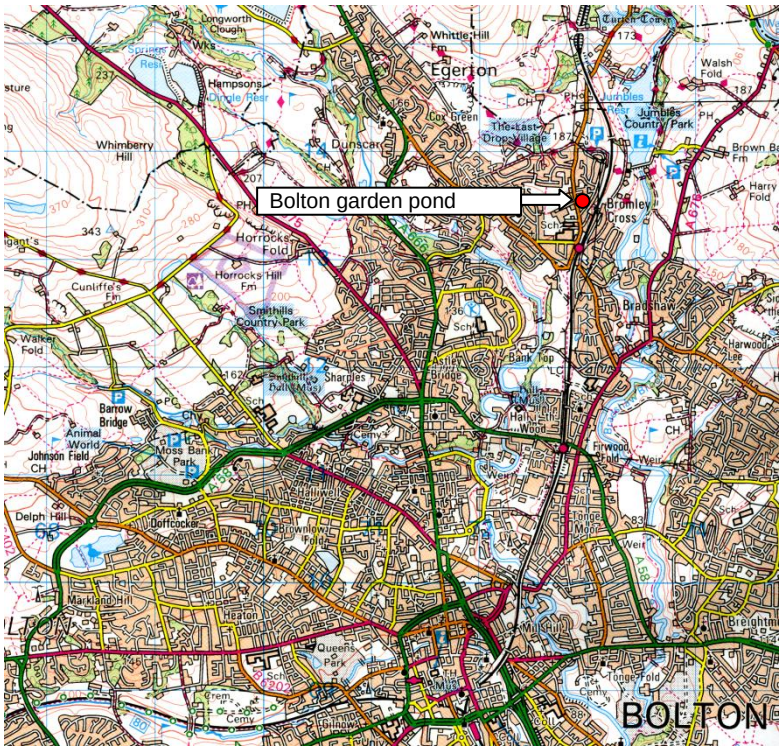
Acorn Bank, east of Penrith, Cumbria



Map 1: Location of Acorn Bank.

Aerial photo 1: Location of the pond at Acorn Bank

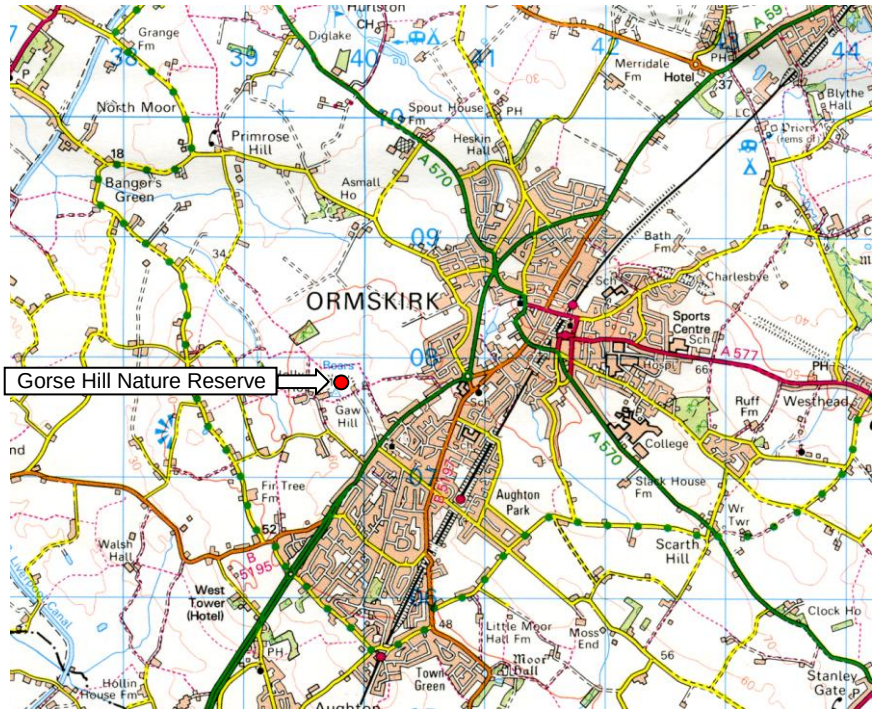
Bolton garden pond, Greater Manchester



Map 2: Location of the Bolton garden pond

Aerial photo 2: Location of the Bolton garden pond

Gorse Hill Nature Reserve, west of Ormskirk, Lancashire



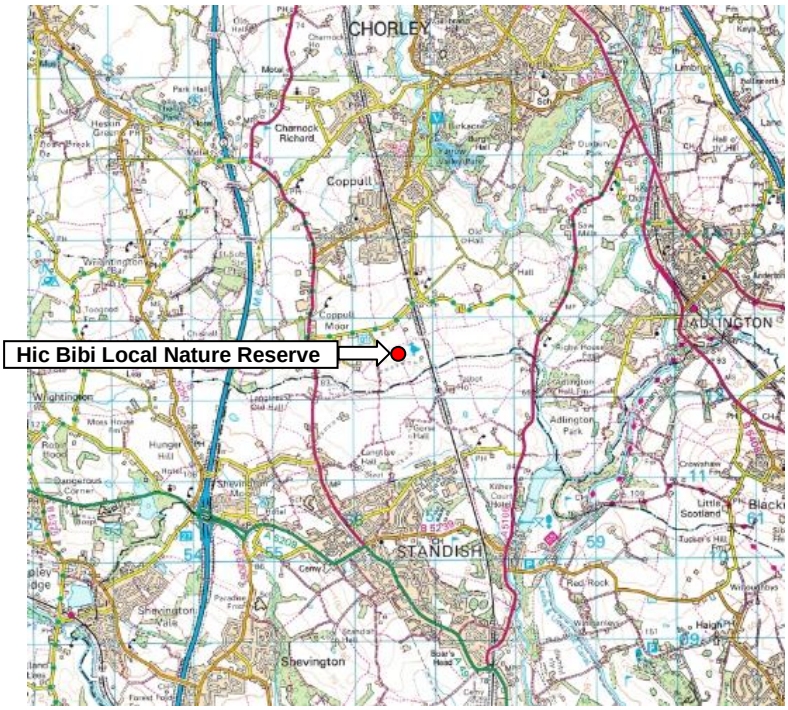
Map 3: Location of Gorse Hill Nature Reserve

Pond with *T. cristatus* present

Pond surveyed but no *T. cristatus* present

Aerial photo 3: Location of ponds at Gorse Hill Nature Reserve

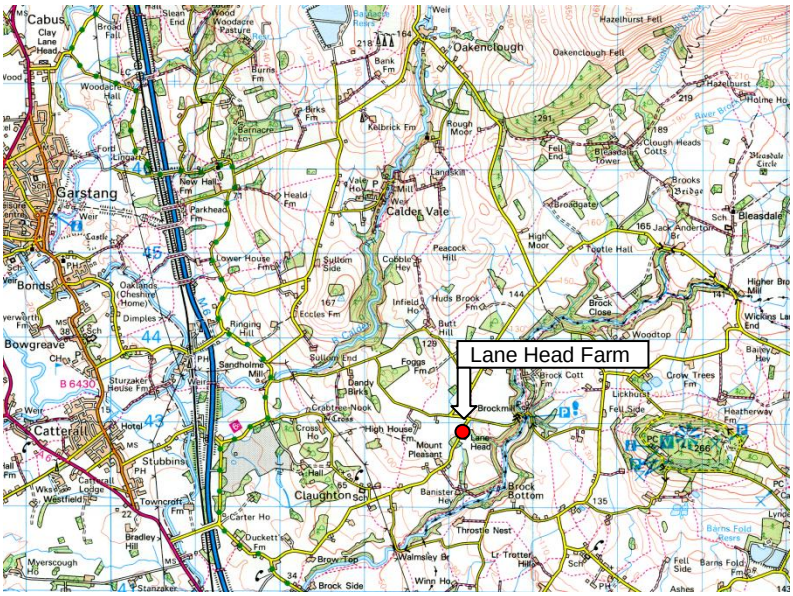
Hic Bibi, south of Chorley, Lancashire



Map 4: Location of Hic Bibi Local Nature Reserve

Aerial photo 4: Location of the pond at Hic Bib Local Nature Reserve

Lane Head Farm at Claughton-on-Brock, south east of Garstang, Lancashire



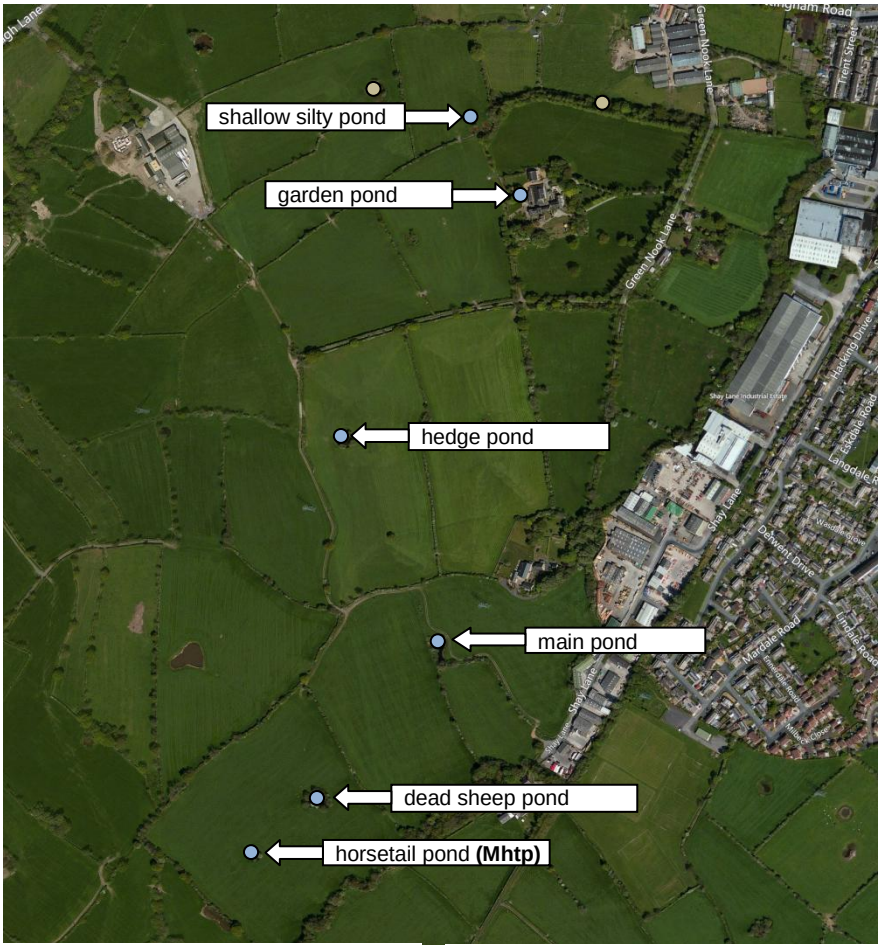
Map 5: The location of Lane Head Farm

Pond with *T. cristatus* present Pond surveyed but no *T. cristatus* present

Aerial photo 5: Location of ponds at Lane Head Farm

Marlings, Longridge, Lancashire

Map 6: The location of Marlings

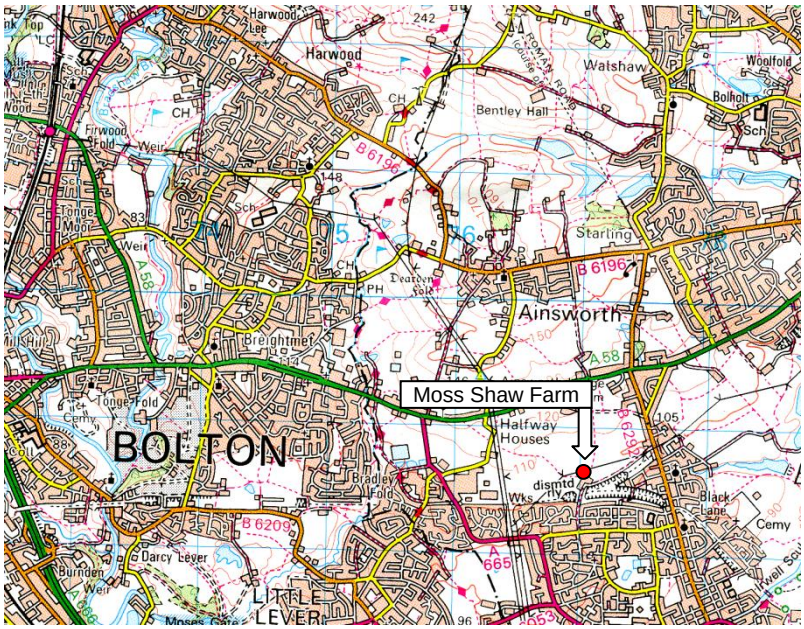


Pond with *T. cristatus* present

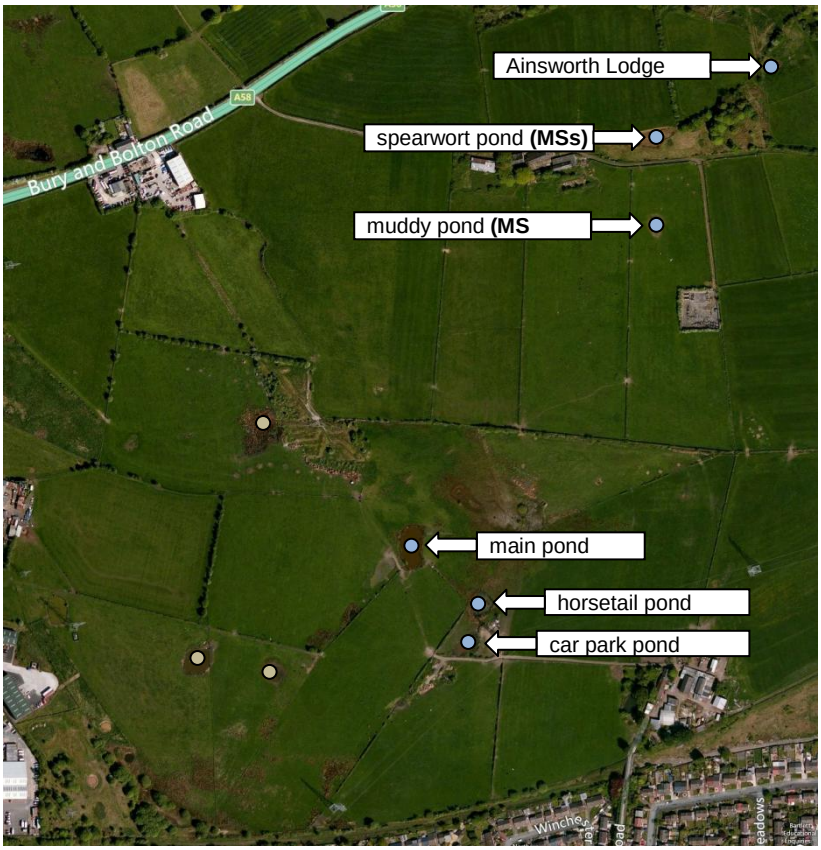
Pond surveyed but no *T. cristatus* present

Aerial photo 6: Location of ponds at Marlings

Moss Shaw Farm, Bury, Greater Manchester



Map 7: The location of Moss Shaw Farm

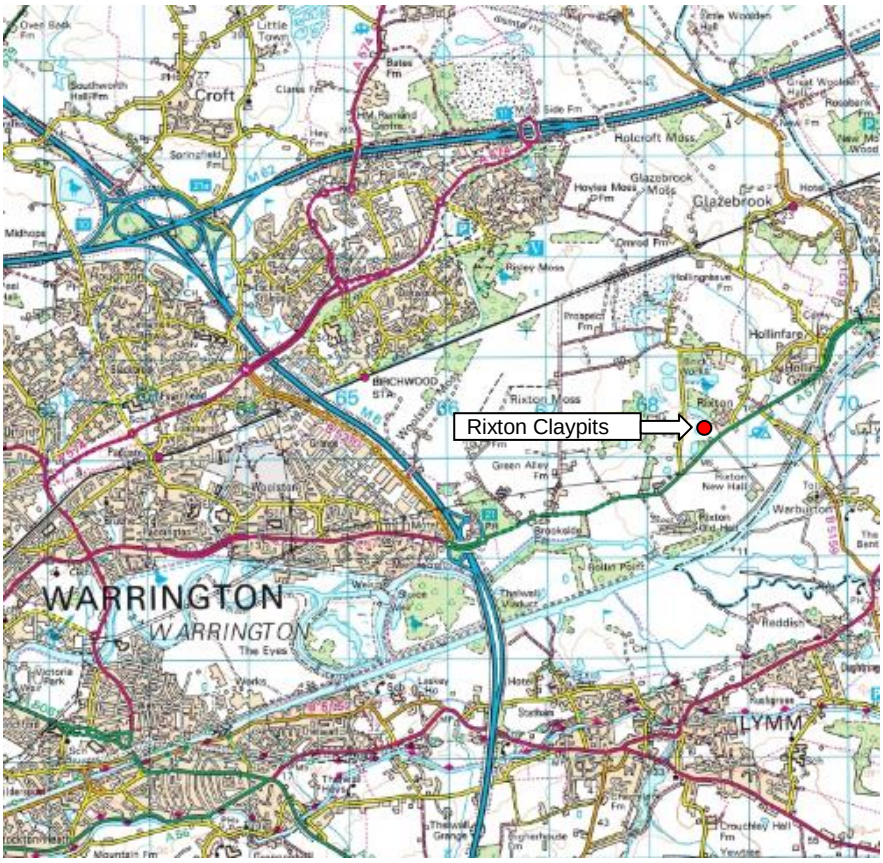


Pond with *T. cristatus* present

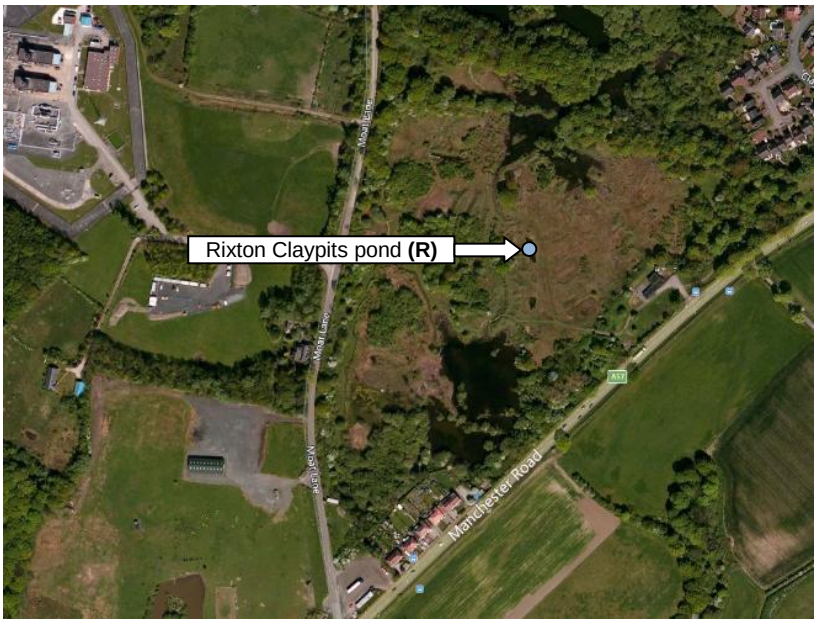
Pond surveyed but no *T. cristatus* present

Aerial photo 7: Location of ponds at Moss Shaw Farm

Rixton Claypits, east of Warrington, Cheshire



Map 8: Location of Rixton Claypits



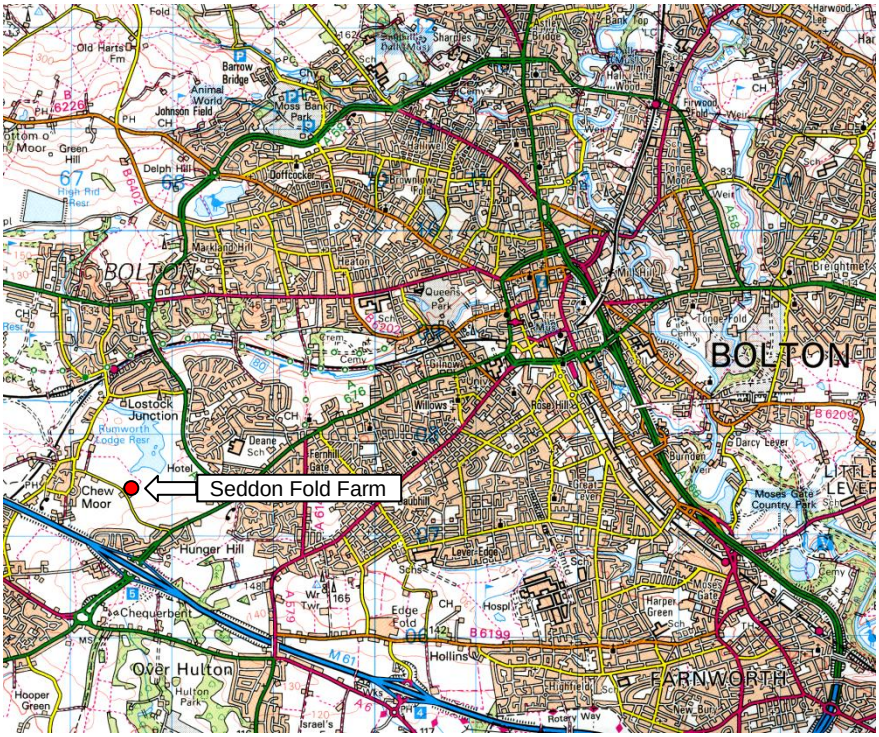
Aerial photo 8: Location of the pond at Rixton Claypits

Raven Crag, west of Penrith, Cumbria

Map 9: Location of Raven Crag

Aerial photo 9: Location of the pond at Raven Crag

Seddon Fold Farm, Westhoughton, Greater Manchester



Map 10: Location of Seddon Fold Farm

Pond with *T. cristatus* present

Pond surveyed but no *T. cristatus* present

Aerial photo 10: Location of ponds at Seddon Fold Farm

Wittlestone Head, south east of Darwen, Lancashire

Map 11: Location of Wittlestone Head

Aerial photo 11: Location of pond at Wittlestone Head

Appendix 2: Photographs of study sites

Acorn Bank pond (AB)

Bolton garden pond (Bgp)

Gorse Hill Nature Reserve (GH)

Lane Head main pond (LH)

Lane Head neighbour's pond (LHn)

Lane Head pond south of road (LHs)

Marlings (Marl)

Marlings hedge pond (Mhp)

Moss Shaw (MS)

Seddon Fold Farm (SF)

Wittlestone Head (WH)

Appendix 3: Plants found at the study sites in 2013

Pond reference	LH	LHS	LHss	LHn	LHhh	Marl	Mhp	Mdsp	Mhtp	MS	MShtp	MScpp
Plant species name												
Agrostis stolonifera	F	O	O	F	F	A	A	F	F	F	A	F
Alisma plantago-aquatica										F		
Angelica sylvestris			O									
Bidens cernua		O								O	O	F
Caltha palustris			F									
Cardamine pratensis		R			O							
Crassula helmsii										O		
Deschampsia cespitosa			F	F	O							
Eleocharis palustris									F		O	F
Epilobium hirsutum											O	
Epilobium obscurum										R		
Epilobium tetragonum		O										
Equisetum fluviatile	O						F		D	A	D	
Equisetum palustre		O									O	O
Galium palustre	O	F	O	F	O							
Glyceria fluitans					O					O		
Juncus acutiflorus		F										
Juncus articulatus		O									O	F
Juncus bufonius agg.												F
Juncus bulbosus		R										O
Juncus conglomeratus										O		
Juncus effusus	F	A		F	A			F	O	F	O	F

(continued over)

Appendix 3 (continued)

Pond reference	LH	LHS	LHss	LHn	LHhh	Marl	Mhp	Mdsp	Mhtp	MS	MShtp	MScpp
Plant species name												
Junus inflexus	O								R		O	
Lathyrus paulstris		O										
Lotus pedunculatus	O	O							R			
Lycopus europaeus									F		F	
Lythrum salicaria		F										
Mentha aquatica	F		O									
Myosotis scorpioides	O	A		O	F				O			
Oenanthe crocata			A									
Phalaris arundinacea	F		A	A			F					
Ranunculus flammula		F			O							
Ranunculus hederaceus				O								
Ranunculus omiophyllus					O							O
Ranunculus sceleratus												O
Rorippa nasturtium-aquaticum	O			O	R				R			O
Rorippa paulstris												O
Solanum dulcamara		A	F	O			F	O			O	O
Sparganium erectum		F					F			F	O	A
Stellaria uliginosa												O
Typha latifolia								A			O	A
Veronica beccabunga	O	O	O	O								
Lemna minor	O	O	F		O		O		O	F	F	F
Lemna minuta									O			
Lemna trisulca											O	F
Callitriche sp (undet.)		O			O		O			O	O	O
Potamogeton pusillus		O					A			A		A

Appendix 4: Invertebrates found at the study sites in 2013including PSYM score.

Pond reference		LH	LHe	LHss	LHn	LHhh	Marl	Mhp	Mdsp	Mhtp	MS	MShtp	MScpp
Invert common name	**Family**												
Beetle	Haliplidae	2			1		2	17		6	3	1	5
	Hygrobiidae												1
	Dytiscidae	7	7	6	9	1	35	5	5	11	2	12	4
	Gyrinidae	1		2									
	Hydrophilidae	9	3	4	16	7	8		2	8	1	11	
Alderflies	Sialidae	9	2				4	1					1
Caddisflies	Limnephilidae							1					
True Flies	Chironomidae	1	4	6	4	1	3	1	7	3	1	1	
Snails	Lymnaeidae							1			1	2	
	Physidae												9
	Planorbidae	1	5			1		8		3	7	1	6
Limpets and Mussels	Ancylidae												
	Sphaeriidae	6	1			2		5	4	4		1	
Worms	Oligochaeta		1		1		1		1				
Leeches	Glossiphoniidae	5	5		2	2	2			1	1		2
	Erpobdellidae					1	2			1			
Crustaceans	Asellidae	4			2		12	8	7	12	4	6	1
	Gammaridae			1		1	1	3			1	3	1
Mayflies	Baetidae	2	8		6	4	10	1		2	4		1
Damselflies	Coenagriidae							11			1		
Dragonflies	Aeshnidae	4			1		2	1			1		

(Continued over)

Appendix 4 (continued)

Pond reference		LH	LHe	LHss	LHn	LHhh	Marl	Mhp	Mdsp	Mhtp	MS	MShtp	MScpp
	Libellulidae							1					
Bugs	Hydrometridae	1	1				3					2	
	Gerridae	13	2	1	4		1	1		1	1	2	4
	Nepidae									1			1
	Notonectidae	5	4				5	1		5	3	2	9
	Corixidae	28	16	46	46	7	17	3	27	4	23	6	19
Total number of groups		**16**	**13**	**7**	**11**	**10**	**16**	**17**	**7**	**14**	**15**	**13**	**14**
Index of biotic integrity		**61**	**67**	**56**	**50**	**39**	**50**	**61**	**28**	**33**	**56**	**50**	**72**
PSYM score		**M**	**M**	**M**	**M**	**P**	**M**	**M**	**P**	**P**	**M**	**M**	**M**

Appendix 5: Dates of all capture visits for all sites.

Site name	Visit number	Date	Total captures
AB	1	29.3.15	99
	2	6.4.15	174
	3	26.4.15	122
	4	3.6.15	126
	5	24.6.15	64
Bgp	1	17.3.12	6
	2	18.3.12	2
	3	19.3.12	15
	4	28.3.12	7
	5	3.4.12	6
	6	11.4.12	12
	7	2.5.12	13
	8	21.5.12	12
	1	31.3.13	2
	2	14.4.13	1
	3	10.5.13	24
	4	22.5.13	11
	5	29.5.13	11
	6	5.6.13	13
	7	3.7.13	4
	8	16.7.13	8
	9	24.7.13	8
	1	30.3.14	1
	2	7.4.14	11
	3	16.4.14	5
	4	20.4.14	9
	5	23.4.14	2
	6	28.4.14	1
	7	7.5.14	12
	8	30.5.14	8
	9	4.6.14	1
	1	6.3.15	1
	2	13.3.15	2
	3	20.3.15	1
	4	3.4.15	9
	5	10.4.15	10
	6	18.4.15	3
	7	24.4.15	10
	8	30.4.15	3
	9	8.5.15	6
	10	15.5.15	5
	11	24.5.15	5

Site name	Visit number	Date	Total captures
Bgp	12	29.5.15	5
(cont)	13	5.6.15	10
	14	12.6.15	6
	15	19.6.15	4
	16	26.6.15	5
	17	3.7.15	8
	18	17.7.15	1
	1	2.4.16	3
	2	6.4.16	6
	3	8.4.16	10
	4	9.4.16	6
	5	15.4.16	6
	6	22.4.16	9
	7	8.5.16	13
	8	13.5.16	13
	9	20.5.16	10
	10	29.5.16	13
	11	10.6.16	13
	12	31.6.16	6
	13	8.7.16	5
	14	17.7.16	5
	15	29.7.16	2
	16	5.8.16	1
GH	1	3.5.13	46
	2	23.5.13	12
	3	7.6.13	15
	4	11.7.13	22
	5	30.7.13	3
	1	31.3.14	14
	2	17.4.14	11
	3	15.5.14	16
	4	16.5.14	14
	5	13.6.14	4
	1	15.3.15	4
	2	4.4.15	40
	3	16.4.15	79
	4	25.4.15	99
	5	2.5.15	115
	6	7.5.15	113
	7	26.5.15	124
	8	22.6.15	36
LH	1	25.4.13	9

Appendix 5: Dates of all capture visits for all sites (continued).

Site name	Visit number	Date	Total captures
LH (cont)	2	4.5.13	20
	3	13.5.13	27
	4	2.6.13	10
	5	27.6.13	6
	6	6.7.13	13
	7	15.7.13	9
	8	16.7.13	18
	9	26.7.13	2
	1	13.4.14	36
	2	4.5.14	14
	3	22.5.14	17
	4	23.5.14	11
	5	24.5.14	13
	1	30.3.15	1
	2	19.4.15	7
	3	5.5.15	23
	4	14.5.15	15
	5	21.5.§5	7
	6	2.6.15	5
	7	9.6.15	11
	8	16.6.15	12
	1	19.4.16	13
	2	7.5.16	25
	3	19.5.16	18
	4	23.5.16	22
	5	30.5.16	24
	6	11.6.16	30
LHs	1	25.4.13	7
	2	12.5.13	4
	3	2.6.13	3
	1	5.5.14	5
	2	23.5.14	2
	3	24.5.14	7
LHn	1	5.5.15	2
	2	14.5.15	2
	3	21.5.15	7
	4	2.6.15	1
	5	9.6.15	1
	1	23.5.16	9
	2	30.5.16	5
	3	11.6.16	6

Site name	Visit number	Date	Total captures
Marl	1	17.5.13	42
	2	30.5.13	22
	3	12.6.13	9
	4	10.7.13	3
	5	24.7.13	2
	1	15.4.14	27
	2	7.5.14	9
	3	8.5.14	23
	4	29.5.14	8
	1	14.4.15	28
	2	15.4.15	34
	3	28.4.15	4
	4	17.5.15	2
	5	27.5.15	7
	6	11.6.15	17
	7	17.6.15	4
Mhp	1	27.4.13	5
	2	7.5.13	8
	3	17.5.13	6
	4	30.5.13	4
	5	12.6.13	3
	6	10.7.13	1
	1	7.5.14	12
	2	8.5.14	9
MS	1	29.4.13	2
	2	11.5.13	8
	3	24.5.13	4
	4	27.5.13	2
	5	6.6.13	22
	6	4.7.13	6
	7	12.7.13	1
	1	10.4.14	14
	2	22.4.14	1
	3	3.5.14	18
	4	18.5.14	25
	5	3.6.14	2
	1	18.5.15	20
	2	23.5.15	4
	3	4.6.15	20
	4	8.6.15	14
	5	15.6.15	14

Appendix 5: Dates of all capture visits for all sites.

Site name	Visit number	Date	Total captures
MS	6	20.6.15	3
SF	1	23.4.13	18
	2	6.5.13	20
	3	12.5.13	9
	4	20.5.13	6
	5	28.5.13	6
	6	3.6.13	2
SF	7	30.6.13	7
	8	19.7.13	1
	1	30.3.14	3
	2	11.4.14	16
	3	28.4.14	7
	4	17.5.14	15
	5	2.6.14	0
	1	7.4.15	7
	2	13.4.15	11
	3	22.4.15	5
	4	29.4.15	4
	5	10.5.15	5
	6	13.5.15	14
	7	26.5.15	23
	8	31.5.15	19
	9	10.6.15	15
	1	14.4.16	18
	2	26.4.16	4
	3	2.5.16	15
	4	16.5.16	18
	5	21.5.16	22
	6	27.5.16	12
	7	5.6.16	12
	8	8.6.16	13
WH	1	13.5.14	23
	2	18.5.14	30
	3	19.5.14	20
	4	5.6.14	11
	1	9.4.15	10
	2	21.4.15	14
	3	30.4.15	10
	4	11.5.15	34
	5	12.5.15	25
	6	18.5.15	25

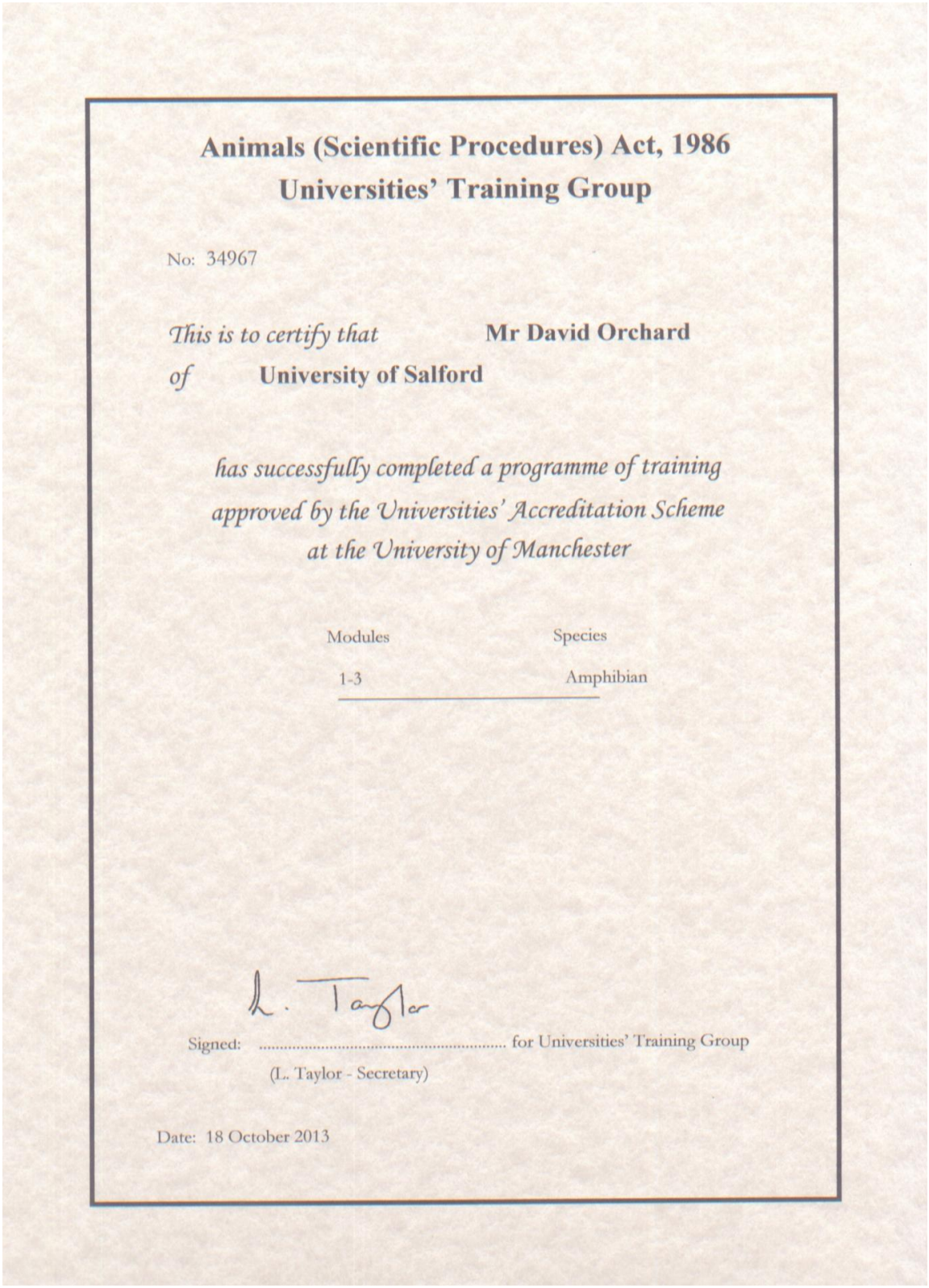
Site name	Visit number	Date	Total captures
WH	8	5.6.15	43
(cont)	9	18.6.15	22
	10	25.6.15	24
	1	15.4.16	6
	2	8.5.16	73
	3	20.5.16	37
	4	24.5.16	67
	5	28.5.16	64
	6	6.6.16	60
	7	14.6.16	46
	8	19.6.16	51

Appendix 6: All adults captured at Bgp 2012-2016

List of ALL names 2012-16	Year of Capture				
	2012	**2013**	**2014**	**2015**	**2016**
Abigail		Abigail			
Aisling2016					Aisling2016
Alfred			Alfred		
Antonia				Antonia	
Beth			Beth		
Cynthia		Cynthia			
Debs_2016					Debs_2016
Edward	Edward				
Eleanor2016					Eleanor2016
Kath			Kath		
Katy O		Katy			
Keith	Keith				
Kim		Kim			
Henry2016					Henry2016
Jennifer				Jennifer	
Liz2016					Liz2016
Nicola2016					Nicola2016
Nigel2016					Nigel2016
Oonagh	Oonagh				
Penny	Penny				
Teresa	Teresa				
Tracy		Tracy			
Sherlock	Sherlock				
Victor	Victor				
Amanda		Amanda	Amanda		
Anne	Anne	Anne			
Cindy	Cindy	Cindy			
Gareth	Gareth	Gareth			
Greg	Greg	Greg			
Helen	Helen		Helen		
John	John	John			
Liz	Liz	Liz			
Louise		Louise	Louise		
Mark	Mark	Mark			
Oscar	Oscar	Oscar			
Samantha	Samantha	Samantha			
Sarah	Sarah	Sarah			
Simon	Simon	Simon			
Stan	Stan		Stan		
Ulrika			Ulrika	Ulrika	
Alison	Alison	Alison	Alison		
Jeremy			Jeremy	Jeremy	Jeremy
Jim	Jim	Jim	Jim		

Lisa Mags Sheena Tina Emily Emma James Lorna Lucy Phil Richard Tasmin Sharon	Mags Tracy Emma Sharon	Mags Tina Emily Emma James Lorna Lucy Phil Richard Tasmin Sharon	Lisa Mags Sheena Tina Emily James Lorna Lucy Phil Richard Tasmin Sharon	Lisa Sheena Emily Emma James Lorna Lucy Phil Richard Tasmin Sharon	Lisa Sheena Emily Emma James Lorna Lucy Phil Richard Tasmin Sharon
Number of capture visits	**8**	**8**	**9**	**15**	**16**
Total number of male captures	12	10	7	4	6
Total number of female captures	14	21	16	11	13
Total captures	26	31	23	15	19
Male population estimate	12.32 +/- 2.33	13.5 +/- 3.49	8.75 +/- 3.92	4.0 +/- 1.0	7.0 +/- 1.08
Female population estimate	17.37 +/- 4.35	27.75 +/- 4.83	22.19 +/- 5.24	11 +/- 2.08	12 +/- 1.58
Total population estimate	28.51 +/- 4.21	42.02 +/- 5.94	31.89 +/- 6.38	14.0 +/- 2.20	19.0 +/- 1.89

Appendix 7: Copy of Home Office personal licence



Animals (Scientific Procedures) Act, 1986
Universities' Training Group

No: 34967

This is to certify that **Mr David Orchard**
of **University of Salford**

has successfully completed a programme of training approved by the Universities' Accreditation Scheme at the University of Manchester

Modules	Species
1-3	Amphibian

L. Taylor

Signed: ..... for Universities' Training Group
(L. Taylor - Secretary)

Date: 18 October 2013

Appendix 8: Allele Frequencies

Tcri 13

	92	94	96	98	100	102	104	106	108	110	112	114	116	118	120	122	124	126	128	130	132	134	138
AL	0.04	0.08	0.04	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.15	0.12	0.00	0.08	0.08	0.00	0.12	0.19	0.00	0.08	0.00	0.00
Bgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.06	0.06	0.22	0.13	0.25	0.08	0.01	0.18	0.00
Bfi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.31	0.13	0.31	0.19	0.00	0.00	0.00	0.00
BFt	0.00	0.04	0.06	0.00	0.10	0.02	0.02	0.02	0.00	0.00	0.04	0.04	0.08	0.04	0.17	0.04	0.00	0.10	0.17	0.06	0.00	0.00	0.02
GHj	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.07	0.02	0.00	0.09	0.27	0.11	0.36	0.02	0.00	0.00	0.00	0.00
GH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.08	0.40	0.07	0.38	0.06	0.00	0.00	0.00	0.00
HBd	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.07	0.00	0.00	0.04	0.00	0.00	0.22	0.22	0.17	0.17	0.07	0.00	0.00	0.01	0.00
HB	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.24	0.03	0.15	0.32	0.00	0.00	0.00	0.00
HBs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.20	0.20	0.00	0.30	0.20	0.00	0.00	0.00	0.00
LH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.09	0.27	0.20	0.25	0.14	0.00	0.00	0.00	0.00
LHs	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.14	0.12	0.19	0.21	0.15	0.02	0.00	0.10	0.00
LHsd	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.14	0.31	0.11	0.19	0.08	0.00	0.00	0.00	0.00
LHss	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.08	0.33	0.17	0.21	0.04	0.00	0.00	0.00	0.00
Mdsp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.16	0.13	0.34	0.06	0.18	0.07	0.03	0.02	0.00	0.00
Mgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.16	0.50	0.18	0.02	0.05	0.07	0.00	0.00	0.00	0.00
Mhp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.33	0.10	0.44	0.03	0.07	0.01	0.00	0.00	0.00	0.00
Marl	0.00	0.00	0.04	0.04	0.00	0.00	0.04	0.00	0.00	0.00	0.04	0.04	0.04	0.27	0.12	0.31	0.00	0.04	0.00	0.00	0.00	0.00	0.00
MR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.07	0.00	0.29	0.21	0.29	0.07	0.00	0.00	0.00	0.00	0.00
MS	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.04	0.01	0.00	0.00	0.07	0.05	0.02	0.01	0.24	0.16	0.25	0.13	0.00	0.00	0.00	0.00
Msmud	0.00	0.00	0.10	0.00	0.05	0.00	0.00	0.00	0.05	0.00	0.05	0.05	0.00	0.05	0.15	0.10	0.00	0.15	0.20	0.00	0.00	0.05	0.00
MSsp	0.00	0.14	0.11	0.04	0.21	0.00	0.04	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.14	0.07	0.00	0.00	0.07	0.00	0.00	0.00	0.00
WHR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.11	0.04	0.01	0.31	0.16	0.36	0.00	0.00	0.00	0.00	0.00
WHR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.42	0.10	0.13	0.00	0.02	0.02	0.10	0.00	0.00	0.00	0.00	0.00

Tcri 27

	244	246	248	252	255	256	260	264	268	272	274	276	278	280	282	284	288
AL	0.00	0.00	0.00	0.06	0.00	0.33	0.33	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00
Bgp	0.00	0.00	0.50	0.04	0.00	0.27	0.18	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bfi	0.04	0.00	0.15	0.00	0.04	0.46	0.08	0.00	0.00	0.00	0.04	0.08	0.00	0.04	0.08	0.00	0.00
BFt	0.11	0.00	0.00	0.05	0.00	0.40	0.16	0.03	0.00	0.03	0.00	0.08	0.00	0.05	0.00	0.11	0.00
GHj	0.03	0.00	0.00	0.03	0.00	0.35	0.24	0.00	0.00	0.09	0.00	0.03	0.00	0.24	0.00	0.00	0.00
GH	0.00	0.00	0.00	0.00	0.00	0.31	0.29	0.00	0.02	0.02	0.00	0.00	0.11	0.26	0.00	0.00	0.00
HBd	0.00	0.00	0.01	0.00	0.00	0.17	0.34	0.01	0.11	0.09	0.00	0.16	0.00	0.09	0.00	0.01	0.00
HB	0.00	0.00	0.03	0.00	0.00	0.47	0.23	0.02	0.09	0.05	0.00	0.11	0.00	0.02	0.00	0.00	0.00
HBs	0.00	0.00	0.00	0.00	0.00	0.27	0.14	0.00	0.05	0.14	0.00	0.41	0.00	0.00	0.00	0.00	0.00
LH	0.00	0.00	0.09	0.05	0.00	0.53	0.23	0.00	0.01	0.05	0.00	0.04	0.00	0.02	0.00	0.00	0.00
LHs	0.00	0.00	0.11	0.02	0.00	0.50	0.23	0.00	0.00	0.05	0.00	0.09	0.00	0.00	0.00	0.00	0.00
LHsd	0.00	0.00	0.00	0.00	0.00	0.97	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHss	0.00	0.00	0.19	0.06	0.00	0.44	0.25	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
Mdsp	0.00	0.02	0.04	0.00	0.00	0.67	0.07	0.00	0.07	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.02
Mgp	0.50	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mhp	0.07	0.00	0.07	0.00	0.00	0.38	0.15	0.03	0.13	0.07	0.00	0.06	0.00	0.03	0.00	0.00	0.00
Marl	0.00	0.00	0.11	0.06	0.00	0.44	0.22	0.06	0.06	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00
MR	0.00	0.00	0.25	0.00	0.00	0.50	0.08	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00
MS	0.13	0.00	0.06	0.00	0.00	0.44	0.20	0.03	0.04	0.00	0.00	0.07	0.00	0.00	0.00	0.02	0.00
Msmud	0.10	0.00	0.00	0.10	0.00	0.60	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MSsp	0.00	0.00	0.00	0.00	0.00	0.50	0.39	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.06	0.00
WHR	0.00	0.00	0.00	0.00	0.00	0.97	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WHR	0.00	0.00	0.02	0.00	0.00	0.36	0.30	0.00	0.29	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Tcri 29

	268	272	276	278	280	284	286	288	292	296	300	304	308	316	318	320	324	326	328	332	336	340
AL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.05	0.00	0.59	0.32	0.00	0.00
Bgp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bfi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.25	0.00	0.00	0.00
BFt	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.56	0.28	0.00	0.00
GHj	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.42	0.00	0.50	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GH	0.04	0.03	0.03	0.00	0.03	0.14	0.00	0.01	0.23	0.01	0.30	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HBd	0.00	0.04	0.04	0.00	0.06	0.07	0.00	0.04	0.23	0.24	0.14	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.00	0.00
HB	0.06	0.07	0.35	0.00	0.18	0.33	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HBs	0.04	0.08	0.23	0.00	0.27	0.23	0.04	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LH	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.02	0.05	0.05	0.00	0.00	0.00	0.03	0.00	0.15	0.47	0.00	0.20	0.02	0.00	0.00
LHs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LHsd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LHss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mdsp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.54	0.25	0.04	0.00
Mgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.03	0.06	0.00	0.00	0.38	0.34	0.00	0.00
Mhp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.04	0.01	0.01	0.81	0.11	0.00	0.00
Marl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.11	0.00	0.78	0.00	0.00	0.06
MR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.33	0.00	0.00
Msmud	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.17	0.75	0.00	0.00
MSsp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.57	0.43	0.00	0.00
WHR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.25	0.50	0.00	0.00	0.00	0.00	0.00
WHR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Tcri 35

	190	198	202	206	210	214	218	221	222	225	226	228	230	234	238	250
AL	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.25	0.00	0.31	0.00	0.16	0.09	0.00	0.00
Bgp	0.00	0.00	0.00	0.00	0.00	0.04	0.48	0.00	0.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bfi	0.00	0.00	0.00	0.00	0.00	0.09	0.18	0.00	0.18	0.00	0.36	0.00	0.09	0.00	0.05	0.00
BFt	0.07	0.02	0.00	0.00	0.00	0.02	0.09	0.00	0.14	0.00	0.39	0.00	0.23	0.05	0.00	0.00
GHj	0.00	0.00	0.00	0.00	0.00	0.02	0.31	0.00	0.31	0.00	0.07	0.00	0.29	0.00	0.00	0.00
GH	0.00	0.00	0.00	0.00	0.00	0.01	0.24	0.00	0.32	0.00	0.08	0.00	0.33	0.00	0.03	0.00
HBd	0.00	0.00	0.02	0.05	0.10	0.03	0.23	0.00	0.23	0.00	0.28	0.00	0.05	0.00	0.00	0.00
HB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HBs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LH	0.00	0.00	0.00	0.00	0.00	0.07	0.10	0.00	0.18	0.00	0.23	0.02	0.13	0.26	0.01	0.00
LHs	0.00	0.00	0.00	0.02	0.07	0.10	0.17	0.00	0.21	0.00	0.05	0.00	0.19	0.19	0.00	0.00
LHsd	0.00	0.00	0.04	0.00	0.00	0.00	0.46	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHss	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.21	0.00	0.64	0.00	0.00	0.07	0.00	0.00
Mdsp	0.00	0.00	0.00	0.00	0.02	0.03	0.20	0.00	0.27	0.00	0.14	0.00	0.27	0.08	0.00	0.00
Mgp	0.00	0.00	0.00	0.00	0.00	0.08	0.11	0.00	0.50	0.00	0.22	0.00	0.00	0.08	0.00	0.00
Mhp	0.00	0.00	0.00	0.00	0.00	0.12	0.27	0.00	0.28	0.00	0.19	0.00	0.06	0.07	0.00	0.01
Marl	0.00	0.00	0.00	0.00	0.04	0.17	0.33	0.00	0.25	0.00	0.13	0.00	0.00	0.00	0.08	0.00
MR	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.08	0.08	0.08	0.17	0.00	0.00	0.08	0.00	0.00
MS	0.00	0.00	0.00	0.00	0.03	0.06	0.15	0.00	0.30	0.00	0.25	0.00	0.17	0.05	0.00	0.00
Msmud	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.23	0.00	0.23	0.00	0.14	0.32	0.00	0.00
MSsp	0.00	0.00	0.00	0.00	0.06	0.03	0.08	0.00	0.22	0.00	0.33	0.00	0.11	0.17	0.00	0.00
WHR	0.00	0.00	0.00	0.00	0.01	0.21	0.05	0.00	0.30	0.00	0.33	0.00	0.10	0.00	0.00	0.00
WHR	0.00	0.00	0.00	0.05	0.11	0.13	0.08	0.00	0.29	0.00	0.21	0.00	0.13	0.00	0.00	0.00

Tcri 43

	260	264	268	272	274	276	280	282	284	288	292	296	300	304	308	316	320
AL	0.00	0.04	0.00	0.04	0.00	0.11	0.25	0.00	0.11	0.32	0.04	0.07	0.00	0.04	0.00	0.00	0.00
Bgp	0.00	0.02	0.43	0.00	0.00	0.03	0.52	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bfi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BFt	0.00	0.00	0.00	0.14	0.00	0.02	0.21	0.00	0.24	0.21	0.02	0.14	0.00	0.00	0.00	0.00	0.00
GHj	0.00	0.29	0.08	0.21	0.00	0.18	0.11	0.00	0.11	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GH	0.00	0.27	0.02	0.32	0.01	0.23	0.09	0.00	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HBd	0.00	0.03	0.08	0.03	0.00	0.18	0.43	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HB	0.00	0.00	0.05	0.09	0.00	0.15	0.40	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
HBs	0.00	0.04	0.04	0.07	0.00	0.25	0.32	0.00	0.21	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LH	0.00	0.00	0.00	0.08	0.00	0.17	0.63	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHs	0.00	0.00	0.02	0.09	0.00	0.22	0.33	0.00	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHsd	0.00	0.00	0.08	0.61	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHss	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mdsp	0.02	0.03	0.15	0.15	0.00	0.41	0.16	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mgp	0.00	0.04	0.00	0.27	0.00	0.42	0.08	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mhp	0.00	0.11	0.10	0.08	0.00	0.08	0.28	0.00	0.34	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Marl	0.00	0.00	0.00	0.17	0.00	0.17	0.17	0.00	0.11	0.06	0.00	0.22	0.00	0.00	0.11	0.00	0.00
MR	0.00	0.07	0.07	0.00	0.00	0.14	0.50	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MS	0.03	0.08	0.04	0.05	0.00	0.16	0.10	0.00	0.05	0.03	0.03	0.35	0.00	0.01	0.01	0.01	0.03
Msmud	0.00	0.00	0.00	0.06	0.00	0.17	0.39	0.00	0.06	0.17	0.00	0.17	0.00	0.00	0.00	0.00	0.00
MSsp	0.00	0.00	0.03	0.30	0.00	0.10	0.23	0.00	0.23	0.03	0.00	0.07	0.00	0.00	0.00	0.00	0.00
WHR	0.00	0.04	0.31	0.25	0.00	0.06	0.12	0.02	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WHR	0.06	0.06	0.06	0.08	0.00	0.06	0.00	0.00	0.14	0.15	0.00	0.31	0.00	0.02	0.00	0.02	0.06

Tcri 46

	272	274	276	280	282	284	286	288	292	294	296	300	301	302	304	308	312
AL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.47	0.00	0.41	0.09	0.00	0.00	0.00	0.00	0.00
Bfi	0.07	0.00	0.00	0.14	0.00	0.04	0.00	0.18	0.21	0.00	0.25	0.07	0.00	0.00	0.04	0.00	0.00
BFt	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	0.00
GHj	0.00	0.00	0.00	0.03	0.00	0.03	0.00	0.00	0.37	0.00	0.05	0.40	0.00	0.00	0.13	0.00	0.00
GH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.31	0.00	0.11	0.38	0.00	0.00	0.13	0.00	0.00
HBd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.28	0.00	0.31	0.21	0.00	0.00	0.10	0.02	0.00
HB	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.05	0.31	0.00	0.26	0.26	0.00	0.00	0.10	0.01	0.00
HBs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.14	0.14	0.43	0.00	0.00	0.11	0.00	0.00
LH	0.03	0.00	0.00	0.00	0.00	0.02	0.00	0.13	0.29	0.00	0.11	0.24	0.01	0.00	0.16	0.01	0.01
LHs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.54	0.00	0.06	0.28	0.00	0.00	0.09	0.00	0.00
LHsd	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.16	0.44	0.00	0.06	0.19	0.00	0.00	0.03	0.00	0.00
LHss	0.08	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.00	0.35	0.00	0.00
Mdsp	0.00	0.00	0.02	0.00	0.00	0.05	0.00	0.15	0.32	0.00	0.10	0.16	0.00	0.03	0.16	0.00	0.00
Mgp	0.00	0.00	0.00	0.06	0.00	0.06	0.00	0.09	0.06	0.00	0.47	0.27	0.00	0.00	0.00	0.00	0.00
Mhp	0.03	0.00	0.00	0.05	0.00	0.04	0.00	0.11	0.25	0.00	0.21	0.23	0.00	0.00	0.08	0.00	0.00
Marl	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.43	0.00	0.29	0.21	0.00	0.00	0.00	0.00	0.00
MR	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.25	0.08	0.00	0.00	0.25	0.00	0.00	0.17	0.08	0.00
MS	0.02	0.01	0.02	0.10	0.00	0.07	0.00	0.10	0.30	0.00	0.24	0.12	0.00	0.00	0.02	0.00	0.00
Msmud	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MSsp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WHR	0.00	0.00	0.00	0.07	0.02	0.14	0.01	0.09	0.28	0.00	0.10	0.05	0.00	0.00	0.24	0.00	0.00
WHR	0.00	0.00	0.00	0.02	0.00	0.07	0.00	0.09	0.28	0.00	0.20	0.20	0.00	0.00	0.15	0.00	0.00

Tcri 50

	174	176	178	180	182	184	186	188	190	192	194	196	198	200	202	204	206	208	210	212	214	215	216	218	220	222	224	226	228	230	240
AL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.08	0.04	0.04	0.00	0.08	0.00	0.33	0.00	0.21	0.04	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00
Bgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.02	0.00	0.14	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bfi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.29	0.00	0.13	0.00	0.04	0.00	0.08	0.00	0.17	0.00	0.04	0.04	0.04	0.04	0.00	0.04	0.00	0.00	0.00
BFt	0.00	0.00	0.00	0.00	0.00	0.05	0.02	0.00	0.00	0.00	0.19	0.00	0.10	0.00	0.10	0.00	0.12	0.05	0.26	0.02	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.02	0.00	0.02	0.00
GHj	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.07	0.02	0.00	0.13	0.00	0.00	0.00	0.35	0.02	0.02	0.00	0.09	0.00	0.04	0.00	0.09	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.21	0.00	0.00	0.01	0.43	0.00	0.01	0.00	0.04	0.00	0.00	0.00	0.17	0.10	0.00	0.00	0.02	0.00	0.00	0.00	0.00
HBd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.01	0.00	0.35	0.00	0.03	0.00	0.03	0.00	0.00	0.00	0.06	0.17	0.03	0.01	0.01	0.00	0.00	0.01	0.01
HB	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.00	0.40	0.00	0.01	0.01	0.04	0.01	0.01	0.00	0.25	0.15	0.01	0.00	0.07	0.00	0.00	0.00	0.00
HBs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.04	0.00	0.42	0.00	0.08	0.00	0.04	0.00	0.00	0.00	0.08	0.21	0.00	0.00	0.04	0.00	0.00	0.00	0.00
LH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.02	0.00	0.30	0.00	0.05	0.00	0.07	0.04	0.10	0.00	0.09	0.14	0.00	0.00	0.01	0.01	0.01	0.05	0.00
LHs	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.37	0.00	0.00	0.00	0.09	0.02	0.00	0.00	0.30	0.13	0.00	0.00	0.04	0.00	0.00	0.00	0.00
LHsd	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.46	0.00	0.04	0.00	0.08	0.00	0.00	0.00	0.04	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LHss	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mdsp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.00	0.01	0.00	0.44	0.00	0.01	0.00	0.08	0.01	0.07	0.00	0.03	0.25	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Mgp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.13	0.00	0.40	0.00	0.03	0.00	0.07	0.00	0.03	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mhp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.11	0.00	0.06	0.00	0.43	0.00	0.01	0.00	0.12	0.00	0.05	0.00	0.02	0.17	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Marl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.39	0.00	0.00	0.00	0.27	0.04	0.12	0.00	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MR	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MS	0.00	0.00	0.17	0.00	0.03	0.06	0.01	0.00	0.14	0.00	0.22	0.01	0.04	0.00	0.08	0.01	0.06	0.03	0.10	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Msmud	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.10	0.00	0.20	0.00	0.05	0.00	0.40	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00
MSsp	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.08	0.00	0.06	0.00	0.31	0.00	0.08	0.00	0.19	0.00	0.03	0.00	0.00	0.19	0.03	0.00	0.00	0.00	0.00	0.00	0.00
WH	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.13	0.00	0.00	0.03	0.32	0.05	0.02	0.00	0.35	0.02	0.07	0.00	0.00	0.00	0.00	0.00	0.00
WHR	0.00	0.02	0.18	0.02	0.00	0.02	0.02	0.00	0.06	0.02	0.20	0.04	0.00	0.00	0.02	0.04	0.06	0.10	0.04	0.04	0.02	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.02	0.00