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Female mate choice preference between three morphologically diverged sympatric cichlid fishes (*Alcolapia* spp.) from Lake Natron, Tanzania

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**Female mate choice preference between three
morphologically diverged sympatric cichlid fishes
(*Alcolapia spp.*) from Lake Natron, Tanzania**



P R I F Y S G O L
B A N G O R
U N I V E R S I T Y

Chloë Nazaneen Robinson

Prof. George F. Turner

Declaration to include in your thesis I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards. -----

----- Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

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Abstract:

African cichlids have often undergone rapid adaptive radiation producing closely related species divergent in morphology, behaviour, and ecology, making them excellent subjects for the study of speciation. *Alcolapia alcalica* (ALC), *A. latilabris* (LAT), and *A. ndalalani* (NDL) are morphologically divergent but genetically similar species which coexist sympatrically in the hot, salty springs and lagoons surrounding soda Lake Natron. This study investigates assortative mating using aquarium mate choice trials, controlling for micro-habitat preference. Trios of males (one per species) were formed and rotated through four tanks, each containing females of one species along with F1 hybrid ALC x LAT. Behavioural observations were recorded and statistically compared to investigate the influence of the presence of conspecific females. Paternity of offspring was determined using microsatellite DNA obtained from skin swabs of mothers and potential fathers and tissues of offspring. It was found that *Alcolapia spp.* in soda Lake Natron mate strongly assortatively, while some heterospecific mating was present within. These results indicate the importance of mate choice in the maintenance of sympatric species distinctness.

Introduction:

Speciation is the process by which ancestral species diverge (Doebeli and Dieckmann, 2000), producing genetic, ecological isolation (Coyne and Orr, 2004), and biodiversity (Wiens and Donoghue, 2004). Within the process of speciation, a single species may diverge into two, encompassing separate narrower ecological niches (Schluter, 2000; Nosil and Feder, 2012); divergence in niche occupation may be accompanied by the acquisition of new heritable traits. The divergence of coexisting sister species may be through an initial shift in a niche, followed by speciation events (Ackerly *et al.*, 2006). There are many processes of speciation: allopatric speciation, divergence through geographic isolation, with individuals adapting to their habitat, which may undergo genetic drift and founder effects, leading to genetic incompatibility (Doebeli and Dieckmann, 2000). Sympatric speciation, species divergence in environments with no geographical barriers (Doebeli and Dieckmann, 2000; Barluenga *et al.*, 2006; Schlieven *et al.*, 1994). Sympatric speciation is controversial as divergence involves other influences such as assortative mating being genotype specific (Schlieven *et al.*, 1994; Doebeli and Dieckmann, 2000). However, in closely related sympatric populations of bacterioplankton, environmental specialisation has been indicated by Hunt *et al.*, (2009) to be a possible influence in previous and current speciation. Suggesting microhabitat preference may be involved in selection with incomplete genetic isolation. In sympatric brood parasitic African indigobirds, *Vidua spp.*, mimicry in host species song and mouth markings have been observed to be used in assortative mate choice, leading to divergent selection (Sorenson *et al.*, 2003).

Ecological diversification is the process whereby individual environmental interactions form barriers, driven by natural selection. This may lead to adaptive traits and thus speciation (Rundle and Nosil; Schluter, 2000; Schluter, 2001). The *Bacillus cereus* group have adapted to novel environments through variation in temperature tolerance, introducing ecological diversity (Guinebretière *et al.*, 2008). The process by which a single genotype adapts to differing environments, producing multiple phenotypes is known as phenotypic plasticity (Pfennig *et al.*, 2010). Pfennig *et al.* (2010) suggest adaptive radiation may be derived from ancestral plasticity events. Ecological divergence can also be a factor in reproductive isolation even when excluding the influence of sexual selective traits such as size (Funk *et al.*, 2006). Chiba (2002) observed that morphological variation between sympatric species does not lead to reproductive isolation; the male gonad size variation in *Mandarina spp.* does not prevent intraspecific reproduction. Habitat variation has been suggested to reduce gene flow in wild populations of *Mandarina spp.* (Chiba, 2002).

Habitat expansion and changes in habitat, introduce the possibility of speciation through colonization of novel environments. The founder effect theory is speciation when a few individuals colonise a new

environment. Whereby, isolation, and variation in selective pressures, lead to new ecological adaptations (Carson and Templeton, 1984; Templeton, 1980). The presence of fragmentation in breeding habitats of *Satyrinae* butterfly species has increased the rate of founder events, decreasing genetic variation (Hill *et al.*, 2005). Isolation is the event whereby a population is divided into two (Hänfling and Brandl, 1998). Isolation events are not completely divided, with gene flow occurring between populations (Hänfling and Brandl, 1998). Bottleneck effects can be created through significant decreases in population. The genetic distance is influenced by the rate at which a population recovers (Harrison, 1991). Deleterious mutations in smaller populations can lead to the acquisition of a new trait and an increased evolutionary rate (Carson and Templeton, 1984; Templeton, 1980). Isolation and environmental differences promote species divergence, Barton and Charlesworth (1984) suggests that there is little evidence that forms a major role for founder effects and bottlenecks. Harrison (1991) argues that these events do not increase the rate of molecular evolution. In general, the role of genetic drift in speciation cannot be ruled out, evolutionary biologists are increasingly focussing on the role of selection in speciation.

Speciation - Assortative Mating:

Assortative mating behaviour is whereby an individual selectively chooses a mate which shares a phenotypic trait (Green, 2019; Endler and Houde 1995), which may be advantageous in events of habitat selection by increasing population fitness (Smith, 1966). McLean and Stuart-Fox (2014) propose that geographic isolation can introduce assortative mating, through the evolution of new traits due to divergence; female mate preference in the guppy, *Poecilia reticulata* varies geographically (Endler and Houde, 1995). Females will prefer a trait presented in their population due to the benefits associated with that trait (McLean and Stuart-Fox, 2014). Variation in fitness is a driver towards assortative mating, such as size relating to fecundity and brood size (Berglund *et al.*, 1986; Olsson, 1993). Jaw morphology in cichlids suggests adaptive radiation events which have enabled niche occupation (Kocher, 2004). Sympatric species can adapt to variations in environmental conditions, such as *Anolis homolechis* and *A. sagrei* have undergone microhabitat partitioning towards differing thermal conditions (Cadiz *et al.*, 2013). However, niche partitioning, does not directly indicate speciation, with intraspecific *Wendilgarda galapagensis* expressing variations in niche occupation with no speciation.

Further divergence has also been observed in a variation in nuptial colouration in closely related species (Kocher, 2004). However, in extreme environments, live-bearing fish, diverged in selection to offspring size, with an indirect influence on fecundity (Reisch *et al.* 2013). The process by which assortative mating occurs can vary with whether the trait is functional such as body size, or whether these are ornamental and tend to only be presented by a single sex (Servedio, 2015).

Variations in mating behaviour can affect mate choice, thus reproductive success (Akopyan *et al.*, 2017), which may ultimately lead to reproductive isolation and speciation (Akopyan *et al.*, 2017). The

process of speciation, mate choice and assortative mating relies on a species being able to identify conspecifics (Higashi *et al.*, 1999). Behavioural differences, such as territoriality, courtship displays (Ord and Stamps, 2009), sound and odour (Caspers *et al.*, 2009) allow for identification through species-specific cues. Species such as the Hawaiian cricket genus, *Laupala*, differ in communication between interspecific species (Shaw and Parsons, 2002), allowing for male species identification. Wing tailed bats (*Saccopteryx bilenata*) show a preference towards the same species wing sac scent, in contrast to their sympatric sister species *S. leptura* (Caspers *et al.*, 2009). The role of sexual selection can have a direct influence on mate recognition, and lead to rapid rates of species divergence (Panhuis *et al.*, 2001). Svensson *et al.* (2010) suggest that sexual selection on morphology is important in the adaptive radiation and speciation in the damselfly, rather than natural selection. *Pseudotropheus zebra spp.* occur sympatrically, Couldridge and Alexander (2002) observed that colour is one of the only possible variations between species. Sympatric species such as *Phylloscopus*, although morphologically similar, have diverged through song variation (Hinde, 1959). Whereby, females will choose mates based on male signals, also observed by Akopyan *et al.* (2017) in female red-eyed treefrogs, *Achalychnis callidrya*. Within species such as cichlids which have undergone rapid rates of adaptive radiation, species recognition has been important in speciation. Plenderleith *et al.*, (2005) identified that when olfactory signals were present between two closely related sympatric species of Lake Malawi cichlids, females showed a significant preference towards conspecific males. Olfactory cues have been used as a form of communication with offspring within cichlid broods during paternal care (deCaprona, 1980). This form of communication is likely not to be present within non-brooding cichlids, however, would indicate a method of species identification with sympatric species. Species-specific cues may also integrate towards sexual imprinting (Verzijden and ten Cate, 2007). Hinde (1959) suggests that bird song variation would have a variation at the genetic level.

Mate choice is the process by which trait expression lead to non-random mating and reproductive investments in conspecifics (Edward and Chapman, 2011). Reproductive isolation is stated by Hendry *et al.* (2000) to be a by-product of adaptation to new ecological niches through the presence of selective behaviours. Hendry *et al.* (2000) compared the allelic variation between the river and beach sockeye salmon; these populations were significantly different, observing a mate choice advantage in beach populations when river immigrants were present, suggesting both populations may have different phenotypic mate choice preferences, which may have influenced genetic divergence (Hendry *et al.*, 2000). Mate choice such as that of the sockeye salmon may result in sterile offspring if mating occurs between different populations (Reiseberg and Willis, 2007). Reiseberg and Willis, (2007) suggest that the incomplete isolation in plant species introduces interspecific gene flow. If the presence of gene-flow is high enough between two populations, this can lead to population homogenisation, and being classified as a single population (Feder *et al.*, 2012; Zaccara *et al.*, 2014). The divergence and

acquisition of different traits within a population, also known as divergent selection, can aid in reproductive isolation (Servedio *et al.*, 2011).

Causes of Mate choice:

Mate choice can arise from mating cost avoidance (Chapman *et al.*, 2003). The higher energetic cost involved in female reproduction, with the inclusion of limited eggs, leads to the assumption that female mate choice is a key driver towards sexual selection (Justus and Mendelson, 2018; Bateman, 1948). Males often make advances towards females regardless of size and colouration (Bateman, 1948; Pyron, 2003). However, females will choose to either accept or reject their advances. Although this is the primary assumption, other research has indicated that other factors such as territoriality, nest building, and courtship displays can be male energetic constraints during reproduction (Justus and Mendelson, 2018); these energetic costs introduce male choosiness in *Betta splendens* (Justus and Mendelson, 2018). The male fruit fly, *Drosophila melanogaster* have an increased cost in male courtship and copulation, whereby males will choose larger females who have an increased fecundity (Byrne and Rice, 2006), suggesting that the energetic costs between males and females during reproduction influence reproductive strategy. Justus and Mendelson (2018) observed male Siamese fighting fish, *Betta splendens* protecting the eggs after fertilisation and building nests. Females showed little preference between the allopatric *Betta imbellis* or their conspecifics. In contrast, the male *Betta splendens* showed a preference towards conspecifics (Justus and Mendelson, 2018), suggesting that assortative mating may be facilitated with only one choosy sex.

Social learning can also influence mate choice success. Hesse *et al.* (2016) observe that depending on the species, isolation can impact the social interaction between conspecifics. However, the results are species-dependent with some individuals showing a decrease in courtship, in comparison to *Betta splendens*, which show an increase in aggression towards intraspecific males after isolation, in comparison to non-isolated. Interspecific aggression has been suggested to be involved in the final stages of divergence, whereby it supports parapatry through competitive exclusion, while also being key to species identification (Nevo *et al.*, 1975). Social hierarchies have a role in male maturity and mating success (Constanz, 1975). In the family *Poeciliidae*, males show strong territoriality, through acts of aggression and bright colouration, in comparison to their dull and smaller male conspecifics (Constanz, 1975). In Lake Victoria cichlid *Pundamilia nyererei*, females will choose based on male colouration, however, territoriality is a secondary factor, important in mating success (Maan *et al.*, 2004). Colour polymorphism in male haplochromines, is under directional sexual selection with intraspecific morphs (Maan *et al.*, 2004). Social interactions, which may not have a genetic basis towards speciation, may also be drivers in conspecific recognition and assortative mating (Hochberg *et al.*, 2003).

Male size can improve mating success, sexually selected by some female species (Partridge *et al.*, 1987). Size preference is a trade-off from benefits associated with male size (Partridge *et al.*, 1987; Censky, 1997). This has been observed in the non-territorial lizard, *Ameiva plei*, with females selecting larger males which provide protection (Censky, 1997). Both sexes may also choose larger mates, as observed in pipefish, *Syngnathidae typhle* (Berglund *et al.*, 1986; Rolán *et al.*, 1999; Bisazza and Marconato, 1988). The size and number of eggs are positively correlated with female size (Berglund *et al.*, 1986). Energetic provision by males to offspring also increased in relation to male size (Berglund *et al.*, 1986). This is also observed in male sand lizards, *Lacerta agilis*, with female fecundity being dependent on size (Olsson, 1993). The polygynous and courtship behaviours of sand lizards constrain courtship frequency (Olsson, 1993). Leading to males choosing larger females, to benefit from the reproductive advantages mentioned prior (Olsson, 1993). Which may suggest that mating strategies such as polygyny and resource control, can skew energetic investments towards males.

Causes of sexual selection:

Species in which male colouration is less ecologically differentiated and more genetically similar may suggest selection through male colouration. (Ribbink *et al.*, 1983; Allender *et al.*, 2003; Knight and Turner, 2004), with polymorphic morphs having an increase in the probability of speciation (McLean and Stuart-Fox, 2014). Dugatkin (1996) observe that genetic and social learning such as imitation can influence female mate choice towards male colouration. When there is a large difference in the male guppy, *Poecilia reticulata* colour intensity, females will show a heritable preference towards brighter males (Dugatkin, 1996). However, it was also observed in this study by Dugatkin (1996), that when colour intensity is low, females will show little preference and imitate social behaviours. Seehausen and Alphen (1998) also observed that Lake Victoria female *Pudamilia nyererei* (*Haplochromis nyererei*) used colouration in interspecific mate choice but when colouration was masked, no assortative mating was observed, with colouration being important in speciation, whereby light conditions can affect the rate of gene flow between two colour morphs (Seehausen and van Alphen, 1998). Species colouration can be an indicator of male fitness and parental quality (Sundberg and Larsson, 1994). Older highly coloured male yellowhammer, *Emberiza cintrinella*, had more offspring, than their younger duller conspecifics (Sundberg and Larsson, 1994); colouration being suggested to be an indicator of paternal quality (Sundberg and Larsson, 1994). However, the ability of the visual system of a species to recognise colouration does affect the influence of colour in mate choice (Seehausen *et al.*, 2008). Conspicuous males increase as these males are preferred by choosy females (Higashi *et al.*, 1999). However, conspicuous males are subject to higher predation., suggesting that the process of sympatric speciation is more likely to occur in barrier-free and predator-free environments (Higashi *et al.*, 1999). Sexual selection can arise through either intrasexual competition or intersexual mate choice (Reichard *et al.* 2005). Sexually selected traits can be introduced with the increase in female preference,

strengthening the presence of a selected trait (Andersson, 1994), such as an increase in tail length in birds or lek size. In bitterlings, *Rhodeus sericeus* dominance is the main influence on reproductive success (Reichard *et al.*, 2005). However, this study by Reichard *et al.* (2005) also observed that when male-male competition is removed, females will choose a male with increased courtship vigour. This secondary trait differed in female preference, as 50% of observations where dominance is present, females chose males which presented increased vigour (Reichard *et al.*, 2005). Hunt *et al.* (2009) suggests that male-male competition can be positive and negative in relation to female attention (Wiley and Poston 1996) when sexual selection is male-driven, this introduces female costs, resulting in dominant male evasion (Chapman *et al.*, 2003).

Genetic correlations between traits can introduce sexual selection (Lande, 1981), with natural selection and sexual selection interacting in mating success (Lande, 1981). Genetic variances in trait preference are maintained through assortative mating, recombination, and polygenic mutation (Lande, 1981). Some species may have genetic predispositions to imprint on specific morphological traits (Verzijden *et al.*, 2005), this would promote associative learning, by supporting sexual selection. Sexual selection may be driven by several factors differing between species. Phenotypic traits may be selected when males lack resource control or paternal care, as stated in the good genes hypothesis (Howard *et al.*, 1994; Ryan and Keddy-Hector, 1992). This hypothesis states that females choose by selecting male genetics. The 'good genetics hypothesis' predicts that trait preferences coevolve (Ryan and Keddy-Hector, 1992). However, the sensory exploitation hypothesis suggests that a genetic preference for a structure may pre-exist in the presence of a species. The ancestor of the sworded swordtail, initially showed a preference for a swordlike structure, prior to the trait presence (Basolo, 1990). Genetic variation on a selected trait may lead to sexual selection, driven by a pre-existing preference (Basolo, 1990). Further research into this sensory bias highlighted that female *Priapella olmecae* preferred males with longer swords. Other species such as guppies show a sensory bias towards the colour orange. Rodd *et al.* (2002) observed that female guppies, *Poecilia reticulata* gravitate towards inanimate orange objects. Detection of food may have influenced this preference, due to the presence of cabrehash fruit in some populations. Suggesting that this orange preference by both sexes may influence sexual selection through environmental factors such as diet and microhabitat, with females sexually selecting traits based on colouration and acquired traits; with males presenting more orange colouration being selected (Rodd *et al.*, 2002). This sensory bias does not directly assist in conspecific selection. *Evarcha culicivora* preferred prey are female mosquitoes which carry blood, allowing for indirect consumption of vertebrate blood (Cross *et al.*, 2009). A mate choice test observed females sexually selecting males who have a blood-based diet (Cross *et al.*, 2009). However, females will not selectively choose a conspecific when both are fed a blood-based diet (Cross *et al.*, 2009), suggesting diet is a driver towards female sexual selection. In contrast, reproductive strategies such as mouthbrooding in some cichlid species can result in the exploitation of sensory bias. The evolution of egg-spots on male anal fins has

been suggested to be correlated to female fitness decreasing with missed eggs (Tobler, 2006). Although egg spots do not have any benefit for the female, Tobler (2006) states that this trait would be maintained due to the fitness benefits of female egg detection. However, there are costs associated with the initial acquisition and costs introduced through selective constraints (Arnqvist, 2006).

Speciation in Cichlids:

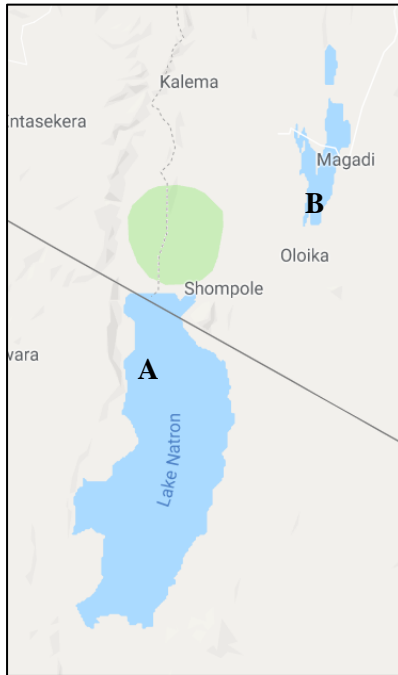


Figure 2. Map of Lake Natron (A) and Lake Magadi (B). Image from Google Images, 3/03/2019.

The family Cichlidae inhabit the Great African Lakes, crater lakes and even extreme volcanic, hypersaline and alkaline lakes. Cichlids have undergone rapid rates of adaptive radiation, which introduce novel forms of phenotypic and ecological diversity (Kornfield, 1978). Adaptive radiation increases species diversity often through the colonization of new environments (Hudson *et al.*, 2010). Greater Lakes, such as Lake Victoria have ~500 endemic species which diverged within an extremely short time (Maan *et al.*, 2004). From a single colonization event, a single species has diverged to 9 morphologically distinct endemic species in a volcanic crater lake in Cameroon (Schliewen *et al.*, 1994). Smaller lakes are useful in understanding the process of ecological divergence (Kavembe *et al.*, 2016), due to the African Great Lakes, having high species richness and rapid rates of adaptive radiation (Kavembe *et al.*, 2016). Lake Natron (Figure 1) is a thermal hypersaline lake in Tanzania. The main water source is the Southern Ewaso Ng'iro river, which originates from Kenya (Kalacska *et al.*, 2017;

Williamson *et al.*, 1993). Lake Magadi and Lake Natron basin separation is estimated to have occurred ~9000 years ago (Kavembe *et al.*, 2016). In contrast to Lake Magadi, Natron is connected to multiple rivers and streams which drain water into the lake (Kavembe *et al.*, 2016), in periods of high rainfall, this may result in water mixing and increased gene flow, not present in Magadi (Kavembe *et al.*, 2016). The lake itself is uninhabitable to fish due to its extreme conditions (Kalacska *et al.*, 2017). The hydrothermal springs which are located around the border (Williamson *et al.*, 1993) and feed the lake are shallow (<3m), pH levels of 10.5, salt >20ppt, temperatures range between 30-42.6 degrees and low dissolved oxygen (0.8-6.46mg/l) (Kalacska *et al.*, 2017; Zaccara *et al.*, 2014). Sodium carbonate deposits are formed, producing crystalline trona covering the main lake (Zaccara *et al.*, 2014). These springs are inhabited by *Alcolapia spp.* cichlids (Kavembe *et al.*, 2016; Zaccara *et al.*, 2014; Williamson *et al.*, 1993). Ancestral mitochondrial haplotypes indicate that the *Alcolapia* flock emerged during the lake separation (Zaccara *et al.*, 2014; Kavembe, 2015).

There are four described species of *Alcolapia*, relating to the morphology and ecology (Seegers and Tichy, 1999) which inhabit two African Lakes. Three occur sympatrically in Lake Natron, *Alcolapia alcalica* (ALC), *A. latilabris* (LAT), and *A. ndalalani* (NDL), with *A. grahami* found in Lake Magadi (Zaccara *et al.*, 2014); *A. grahami* is less morphologically variable, while ongoing research has indicated the presence of upturned mouths within some populations (Kavembe *et al.*, 2016). *Alcolapia* spp. were initially classified within the *Oreochromis* genus by Seegers and Tichy (1999), and then later segregated into their own genus. In further studies, *Alcolapia* has been nested within the *Oreochromis* genus (Trewavas, 1983; Seegers and Tichy, 1999; Nagl *et al.*, 2001; Ford *et al.*, 2019). The nuclear analysis supports *O. amphimelas* being the closest extant sister species (Ford *et al.*, 2019). The adaptation to alkaline lakes is apparent in their small size of maturity, low number of vertebrae and dorsal fin rays (Kavembe, 2014). Behavioural and morphological adaptations to extreme habitats have also been observed in other species such as shorebirds which inhabit saline and alkaline lakes, adapted to obtain their fluid needs from food, alongside adaptations to bill and tongue shape and length (Mahoney and Jehl, 1985). Abiotic factors, such as temperature also affect the reproduction and growth of aquatic plants, (Bornette and Puijalón, 2011). Saline lakes are considered keystone habitats, as endemic and unique species are adapted to thrive in these harsh conditions (Kavembe *et al.*, 2014). Most of the previous research on *Alcolapia* is on the ability of *A. grahami* to survive in extreme habitats and phylogeny. Little is known on Lake Natron *Alcolapia* species divergence and behaviours.

The three soda Lake Natron species are morphologically distinguished by their head shape, mouth shape and teeth number, shape and position (Seegers & Tichy 1999; Kavembe *et al.*, 2016). Male colouration also varies between the three species (Ford *et al.*, 2015). Ford *et al.* (2016) suggest that the lack of predators and competition in Lake Natron, alongside the common tolerance of saline and thermal conditions in *Oreochromis*, would have inhibited the adaptation to the extreme environments of Lake Natron. This lack of competition and predators would have enabled intraspecific divergence towards specialised feeding morphology (Higashi *et al.* 1999; Ford *et al.*, 2016), through the occupation of diverse environments (Kavembe, 2015). ALC is distributed across both Northern and Southern populations. However, NDL and LAT, only inhabit the south of the lake. ALC clades have been observed between the Northern and Southern population, suggesting population substructures (Ford *et al.*, 2015). A population of ALC in the Shompole swamps, located north of Lake Natron have a blue hue, while male ALC in South-Western locations have a darker, purple pigmentation (Kavembe 2015). This colour variation may suggest possible morphological variation between populations.

Although significantly morphologically different, there is little genetic variation between the three Lake Natron species (Ford *et al.*, 2015), suggesting incomplete reproductive isolation in the presence of gene flow (Kavembe *et al.*, 2016; Ford *et al.*, 2015) and interspecific hybridization (Ford *et al.*, 2015). The lake is separated through crystalline trona, connected through heavy periods of rainfall (Zaccara *et al.*, 2014; Ford *et al.*, 2015; Kavembe *et al.*, 2016) and piscivorous birds (Zaccara *et al.*, 2014; Kavembe *et*

al., 2016). Ford *et al.* (2015) observed high levels of interspecific gene flow in Lake Natron, indicated in mtDNA and microsatellite DNA analysis (Kavembe, 2015). Saline fish have been observed to express a variation of morphology, ecology, and behaviour, leading to intra- and interspecific variation even in the presence of gene flow (Kavembe, 2015). Zaccara *et al.*, (2014) and Seggers and Tichy (1999) suggest that the lack of genetic variation in comparison to morphology, relates to the difference in foraging behaviour, enabling resource partitioning, which may have occurred recently and may not be reflected genetically. Barluenga *et al.* (2006) observed sympatric speciation through divergence in behaviour and resource use in Midas cichlids, *Amphilophus spp.*, possibly through events of disruptive natural selection. Chapman *et al.*, (2013) observed that little loci difference between *Senecio spp.* which inhabit differing altitudes of Mt. Edna is sufficient to maintain species phenotypic and ecological divergence even in events of hybridisation.

Microhabitat partitioning has been observed between the three sympatric *Alcolapia spp.*, with benthic LAT and NDL males spending higher frequencies of time on rocks in contrast to ALC (Ford *et al.*, 2016). *Astatotilapia* ecomorphs inhabit different water depths in crater lake and riverine habitats (Malinsky *et al.*, 2015); *Astatotilapia* differ in breeding colour, shape, feeding morphology and diet (Malinsky *et al.*, 2015). It was observed that the *Astatotilapia spp.* preferred to mate with males which were more genetically similar, with nucleotide diversity differing between benthic and littoral morphs (Malinsky *et al.*, 2015). The geographic morphological and genetic variations have been observed in other species, such as migratory bats, which differ in genetic structure and wing morphology between populations (Miller-Butterworth *et al.*, 2003). In some cichlid species, such as Midas cichlids, *Amphilophus spp.* express differentiation between populations through independent founding lineages, and not through sympatric speciation events (Barluenga and Meyer, 2010). Gene flow may limit species divergence through population homogenisation (Feder *et al.*, 2012; Zaccara *et al.*, 2014). Suggesting assortative mating may be an influence in sympatric speciation facilitated through microhabitat partitioning.

Study System:

As stated previously, it is important to look at the divergence of species in small lakes, to further understanding of adaptive radiation and processes of divergence. In addition, extreme lakes, such as Lake Natron, introduce further questions about what adaptations enable these species to thrive. Sexual selection can be a key driver in speciation. Observing the effects of mate choice and behaviours presented may indicate whether these species are morphologically diverse due to females preferring conspecifics through ecological selection or social interactions. The microhabitat partitioning presented by *Alcolapia spp.* may provide a basis for reproductive isolation (Ford *et al.*, 2015). If assortative mating is present independent of habitat differentiation this may suggest the level of speciation, with mate choice been subject to other behavioural or environmental factors. Investigating conspecifics and

heterospecific behaviours would provide an insight into whether these variations in interactions are drivers in assortative mating, sexual selection and thus influencing sympatric speciation.

ALC populations are considered endangered, while NDL and LAT are likely to have an increase in threat due to their feeding specialisation (Kalacska *et al.*, 2017; Zaccara *et al.*, 2014). The main causes of this decrease in population are climate change and habitat degradation (Kalacska *et al.*, 2017), due to anthropogenic disturbances, such as soda ash mining (Zaccara *et al.*, 2014). This Lake is part of the Ramsar Wetland site, which is unprotected, except for hunting regulations (Kalacska *et al.*, 2017). Research on this species will allow for further understanding of the species behaviours, and the process of divergence, to further comprehend how ecological divergence may occur, observing whether female mate choice is involved in assortative mating. The results would then be transferable to other research on ecological diversification and sexual selection.

Aims:

The purpose of this study is to use microsatellite DNA paternity testing to determine whether assortative mating contributes towards reproductive isolation between three genetically similar *Alcolapia* species from Lake Natron. This will be augmented with behavioural observations, which will indicate the possible influences of female and male- preferences and male-male competition on the experimental results. This study will test the hypothesis that Lake Natron *Alcolapia spp.* mate assortatively independent of habitat preferences, with conspecific males showing higher frequencies in behavioural traits in correlation to conspecific species. Species-specific tanks will be contrasted, whereby male Trio groups (one male of each species) will be rotated once offspring are collected and preserved. Three tanks will be comprised of species-specific females (e.g. Tank 1 *A. alcalica* females), an additional tank will consist of *A.alcalica* x *A. latilabris* hybrid females. Each male will be observed, and behavioural frequencies will be recorded. The male behaviours will be compared across species tanks to investigate any variation presented between conspecifics and heterospecific females. Mother and all males will be swabbed, and offspring will be euthanised. The offspring and parental DNA collections are genetically analysed to configure paternity.

Methods

Experimental species:

The species used in this study were three closely related cichlid fish endemic to Lake Natron, Tanzania. *Alcolapia alcalica*, *A. latilabris*, and *A. ndalalani*, (Figure 2) differ in colouration and mouth morphology, and coexist sympatrically in the hot, hypersaline and hyperalkaline springs and lagoons located around the southern edges of the lake (see Figure 1). All three species are maternal mouthbrooders: the brightly coloured males occupy small territories and dig spawning structure or

bowers in the substrate. The camouflaged females visit male territories to lay eggs, which are then picked up by the females and brooded in the oral cavity until the fry are released.

All the specimens used in this study were first generation (F1), reared in captivity from wild caught parents

Experimental Design:

Males were formed into trios, one of each of the pure-bred species, and presented to a group of 10 females, all of a single species or cross. 6 replicate trios of males were used, and 4 tanks of females (one of each species, plus F1 hybrid *A. alcalica* x *A. latilabris*). It was originally intended to use each trio in

each female tank, providing a balanced design of 24 trials, but one or two of the males seemed to be ill, and it was also found that the fish grew rapidly, and the levels of aggression were becoming higher, so the experiment was stopped after 12 trials, with each trio being used in 2 female tanks.

When not in experimental trials, individual males were kept isolated in 9 litre tanks connected to a 2,000-litre temperature-controlled recirculating system, the water temperature and quality were checked regularly to ensure it meets the environmental needs of fish, stated prior. Six experimental trio groups were formed from a single male of each species. The variation in size (standard length SL, mm) and colour were not taken into consideration when creating male trio groups. Apparent females (large individuals not showing male breeding dress) were housed in groups of 10 in 200L tanks, also connected to the same recirculating system. Due to juvenile males showing no colouration until maturity, the apparent females were checked daily to confirm sex, and any individuals developing male breeding dress were removed from the tank and replaced.

Experimental protocol:

Tank set-up:

Four 200l tanks were prepared using a thin layer ~1cm of play sand as substrate,

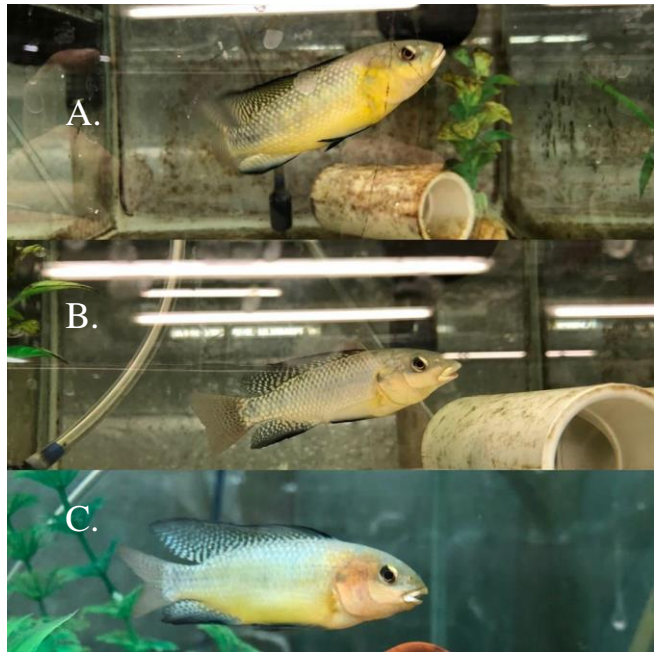


Figure 2. Image of the three experimental species. A. *Alcolapia alcalica*, B. *Alcolapia latilabris*, C. *Alcolapia ndalalani*. Each species differs in colour, size, and jaw morphology.

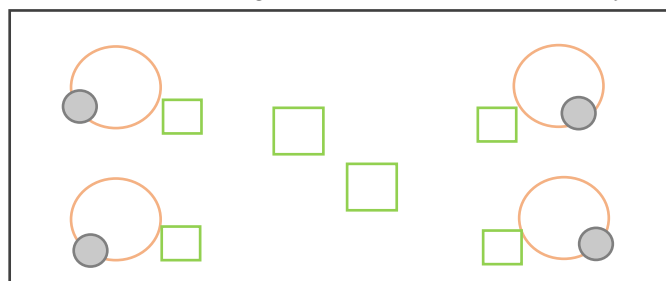


Figure 3. Diagram of tank set up. Four flowerpots (orange) were placed in each corner, alongside a rock (grey) and an artificial plant (green). A play sand substrate was also used (~1cm in depth).

alongside four flowerpots weighed down with rocks and artificial plants, layout reflected in Figure 3. The plants provided a form of distraction, with the possibility of increasing probability of territoriality. Sand and rocks were used to try and mimic their natural habitat, as observed in Figure 4. Providing areas to create bowers, to use in territoriality or to perform courtship displays. Each tank housed females of a single species or hybrid cross (N=10), along with a trio of males, one of each pure-bred species (N=3), following a similar experimental design by Knight et al. (1998).



Figure 4. Image of Lagoon near Lake Natron. Natural Habitat of *Alcolapia alcalica*, *A. latilabris* and *A. ndalalani*. The use of sand and rocks were to imitate this habitat. Image from George Turner- YouTube video of Alcolapia.

Mouthbrooding females were isolated in 9l or 30l tanks. These also included rocks and a mesh flooring, see Figure 5. While mothers of most related species (*Oreochromis*) care of the offspring for several weeks after release from the mouth, it was observed that *Alcolapia* mothers did not do so, and indeed sometimes tried to eat the offspring. The mesh allowed for an area for the fry to hide successfully, but in general the offspring were removed from the mother's mouth and euthanised before release. Rocks were used to weigh down and raise the pseudo-floor observed in Figure 5.

Swabs were taken using sterile medical swabs with a cotton end. This was performed through moving the swab across the body of the fish. The fish were held in a net. The procedure was undertaken rapidly, to prevent long periods of stress from removal of water. This procedure was only undertaken on mothers and all the males. Males were swabbed once during a rotation. Females were swabbed when placing back in the main tank after expelling fry, reducing handling time and stress.

Fry were euthanised using schedule 1 protocol for humane killing indicated in Animals (Scientific Procedures) Act 1986. An anaesthetic (ms222) was added to a small amount of water, mixed with a small amount of bicarbonate soda. Fry were introduced. Once dead, death was confirmed by destruction of the brain. These were then preserved in 100% ethanol and kept in a -8-degree fridge.

Behavioural analysis:

5-minute videos were taken to analyse the proportion of time each male spent expressing the behaviours indicated in Table 1. Focal watches were carried out three times per video focussing on each male, recording duration and frequency of behaviours presented. Behaviours include aggression towards males, aggression towards females, courtship, aggression from other males, interactions of aggression or courtship towards adjacent tanks, digging bowers and feeding on algae or sand, as presented in Table 1.

Using SPSS, a pairwise comparison was performed to observe any variation in behaviours between the female species tanks. Due to violation in Levene's test for normality of variances, the non-parametric Mann-Whitney U test was performed ($p < 0.05$). There are 18 pairwise comparisons that can be made,



Figure 5. Image of the mesh flooring used to prevent mothers attacking fry if expelled in the tank. The mesh was held down by rocks. This flooring was set up in 16, 9 litre tanks.

categorised into one of the following: Males with conspecific and heterospecific females, males in with two different heterospecific females, Males in with conspecific and hybrid females, and males with heterospecific and hybrid females. Quantitative analyses focussed on AGA, AGL, AGN, CB, and T (Table 1).

Table 1. Ethogram of behaviours which were observed during ad lib observations. Each focal male was observed for the full duration (5min). When a behaviour was observed, the duration (seconds) and frequency was recorded.

| Code | Behaviour | Description |
|-------------|----------------------------|--|
| AGA, | Aggression towards another | Focal male chases, bites or shows any form of aggression towards another male. |
| AGL, | spp. male | |
| AGN | | Fighting between the males is recognised by raising of the dorsal fin and in some cases of the throat, used to make the male look larger. |
| CF | Chasing Female | Male follows or chases an anonymous female. |
| CB | Courtship Behaviour | Focal male moves body on an angle and shakes body. This is performed around a female, and near her face. |
| FA | Feeding Algae | The male feeds on the flowerpots, stones, or flowers. |
| FS | Feeding Sand | Looks for food in the sand. Identified through picking up and dropping food straightaway either stationary or while moving. The location where the sand is picked up is not shallow. |
| D | Digging | The male will pick up sand from an area with little sand and drop the sand away from where it was collected. |
| T | Territorial | Male will stay in a single location (flowerpot or stone) for a long period of time. Defending this area from any other males. |

| | | |
|--------------|---|---|
| AFA , | Aggression from another spp. | Male aggression is directed towards the focal male. |
| AFL , | male | |
| AFN | | |
| NM | No movement | Male stays stationary in one location for a long period of time. This tends to be on the bottom of the tank. |
| IWA | Interaction with adjacent tank – Aggression | Aggression is directed towards a male of another tank. |
| IWC | Interaction with adjacent tank – Courtship | Courtship behaviour is observed towards a female of the adjacent tank. |
| TW | Top Water | Male is gulping at the top of the water. |

- Duration is recorded for all behaviours. Biting and any other instant behaviours are tallied.

DNA extraction:

DNA extraction- in home method:

The DNA extraction procedure was carried out using Schuelke, (2000). Fin clips were taken of the preserved offspring (N=139), using ~half the caudal fin of each fry. This was placed into 15µl of 20mg/mL proteinase K and 400µl DNA extraction buffer; vortexed and incubated at 57°C for 30 minutes (m) or overnight, then centrifuged for 2 min at 11,000rpm spin, removing and discarding any remaining fish parts from the supernatant. 400µl isopropanol was added (pre-chilled at -20°C), and the tube inverted 3-5 times and placed into a -80°C freezer for at least 10 min. Once defrosted, the liquid was centrifuged for 10 min at full speed (4600rpm) to form a pellet, which was dried by pouring away the solution onto a paper towel. This was then cleansed, by adding 190µl 70% ethanol, flicking the pellet and centrifuged at full speed for 2 min. The pellet was further dried, by removing any liquid using a pipette, then heated in the heat block set at 55°C for 5-10 minutes, until the pellet was fully dry. The pellet was resuspended using 30 µl of elution buffer, heated on a 65°C heat block for 5-10 minutes. A similar DNA extraction method was used for the swabs (N=43, Female =25, Male = 18). However, instead of the initial incubation, initially the swab was placed in 57°C pre-heated DNA extraction buffer for 15 min, and vortexed.

Fluorescent labelling of PCR fragments:

To decrease PCR cost a fluorescent 3 primer tag method was used following Schuelke, (2000). A 10µl PCR 3 primer product was produced, using 4.723µl dH₂O. 2µl 5X Green Go Taq Flexi Buffer, 1.012 µl MgCl₂ (Magnesium chloride) 25mM, 0.22 µl dNTB (Dinitrothiocyanobenzene) 10mM, 0.44 µl forward primer with M13 tail, and 0.44 µl corresponding reverse primer, 0.11 µl fluorescent FAM, PET, VIC, and NED labelled universal M13 primer tag, and 0.055 µl Taq enzyme. All except the

forward and reverse primers were incorporated into a master mix, to increase quantities and reducing variability between primers. 1 µl of DNA, diluted to 1:20 is then added to 9µl PCR product. This was then run for 2h in the PCR. A 2-primer method was also used, whereby the Florescent tag was already incorporated into the forward primer. After a series of testing of multiple primers, four primers were used to analyse the DNA extractions, see Table 2.

Table 2. Primers used in PCR sequencing. These are the four primers which were used, each had a fluorescent tag used in allele identification during peak analysis.

| Sequence | | | |
|------------------------|-------------------------|-----------------|--------|
| Forward Primer | Reverse Primer | Fluorescent Tag | Colour |
| CTGTTTCTTTGCCCAAACGGT | CCAATGACCGTGCTTACAGGA | NED | Yellow |
| TGGAGGATGCGACGCTCATT | CTGTGAAGCGTTTTTCTGGGGTA | VIC | Green |
| ACACAGACGGTCATAATCCCTG | GGAACAGTTTAAGGCACAAGTCC | PET | Red |
| TGTACTAAGCAGCTCCCAGGT | GGTGGAAATACGCACAACGA | FAM | Blue |

Agarose gel:

Gel electrophoresis was used to detect the presence of DNA after the DNA extraction, or the length of DNA between primers in PCR products. The agarose gel was formed of 1g of genetic analysis grade agarose (C₁₂H₁₈O₉), to 100ml of 0.5x Tris-acetate-EDTA (TAE) buffer. This was microwaved for ~2min 30s mixing the solution every 30s, to prevent over-boiling and buffer evaporation, as this may alter final agarose percentage. Once cool, whereby it can be held, 2µl SyberSafe was added, allowing visualisation of DNA via UV light. The solution is then poured into tray with a positioned well-comb, removing any bubbles prior to setting for ~40-60 mins.

The tray is then added to the electrophoresis unit containing the same buffer as used to make the gel, with the buffer covering the top of the gel. 2 µl of loading dye is added to 5 µl of DNA extraction solution. For the PCR, where green buffer was used no loading dye was needed. 2 µl of PCR product or 7 µl of DNA was pipetted into the wells and the corresponding amount of DNA ladder added to the first well, providing an indication of the number of base pairs (bp).

Sequencing:

When using the green Go Taq Flexi Buffer, the green dye was cleansed prior to sequencing. The PCR product is cleansed through adding 80µl of 80% isopropanol to 10µl of PCR product, mixing using a pipette and leaving for 10min, before being centrifuged at 3870rpm for 1hour, creating a DNA pellet. The plate is inverted removing any remaining liquid, before spinning upside-down over a paper towel at 1000rpm for 1min. 100µl of 70% isopropanol was added into each well, spun at 3870 for 10min, discarding any liquid, before spinning upside down as previously and airing for 15 min.

To aliquot the samples for transcription, ~1µl of a FAM, PET, VIC or NED primer PCR product was mixed, see Table 2 for primer and fluorescent tag allocation and colour identification. To prevent the cleaning process, 5X colourless GoTaq Flexi Buffer was used when making the PCR solution. The solution was then ready to be sequenced. This was performed in collaboration with UCL and labs in Germany which assisted in the sequencing of the samples. From the DNA extractions, only selected samples were sent to be sequenced. From Soda 1 N=29, Soda 2 N=8, Soda 3 N=45, and Soda 4 18 fry were selected for analysis. Of the female swabs, Soda 1 N=5, Soda 2 N=4, Soda 3 N=14, Soda 4 N=4 and all Males from each Trio underwent microsatellite sequencing. The sequencing results were analysed using ThermoFisher Microsatellite Analysis Software (MSA). From the MSA software, allele sizes were collected and manually inputted into Excel in the format shown in Appendix 1. Paternity was determined by manual comparison of offspring allele sizes with those of the possible parents. Usually, the maternal alleles were known, and there were only 3 possible fathers (no more than 6 alleles per locus). The two offspring allele sizes for each of the 4 primers mentioned prior were compared against the mother and the three possible fathers. On the rare occasion where the mother's genotype was unknown, due to not being swabbed, the allele size distributions of the potential fathers were sufficiently different to allow unambiguous determination of paternity

Results:

Paternity Results:

Figure 6 indicates the results obtained from the genetic analysis. Not all males were introduced into all female tanks but were exposed to at least two different female species. The result observed in Figure 6, indicate that conspecific males fathered significantly more broods. Heterospecific paternity was present across seven of the nine trials (see Appendix 4).

Table 3. Male trio rotations across the four female tank. Each trio was not introduced to all 4 aquaria; each Trio was introduced to at least two species.

| Species tank | Trio Number |
|---|-------------|
| <i>Alcolapia alcalica</i> Females | 1, 4, 6 |
| <i>Alcolapia latilabris</i> Females | 2, 3 |
| <i>Alcolapia ndlalani</i> Females | 1, 2, 3, 5 |
| <i>Alcolapia alcalica</i> x <i>Alcolapia latilabris</i> hybrids | 4, 5, 6 |

Table 3 indicates the tanks each Trio was introduced to. Figure 6 indicates a higher frequency of paternity towards conspecifics, successful heterospecific paternity varied across tanks and trios, see Appendix 4. ALC males fathered offspring in all species tanks. However, paternity was only observed in one exposure in both NDL and hybrid females. LAT males fathered offspring across

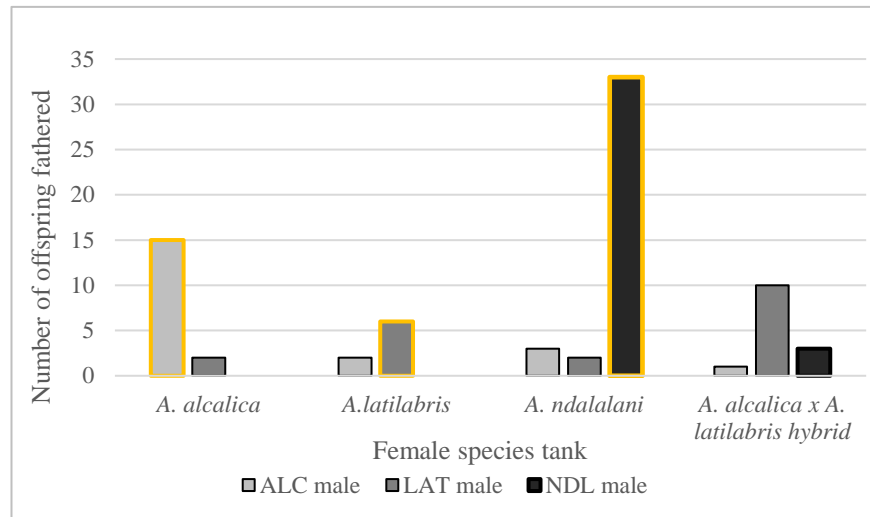


Figure 6. Number of offspring fathered by each male species across the four female species tanks. *A. alcalica* (ALC) females were introduced to male Trios 1, 4 and 6. *A. latilabris* (LAT) with Trios 2 and 3. *A. ndalalani* (NDL) with Trios 1, 2, 3, and 5. The F1 hybrid *A. alcalica x A. latilabris* females were introduced to Trios 4, 5, and 6. Conspecific species (yellow) sired significantly more offspring than heterospecific males. LAT males fathered the most offspring with hybrid females. NDL sired no offspring with ALC and LAT females.

the most exposures. NDL males fathered the least offspring across all trials. The NDL males did not sire offspring within the ALC and LAT tank, while only fathering offspring in one exposure in the hybrid tank.

Across the ALC x LAT hybrid females, there was a higher number of broods fathered by LAT males, see Figure 6, while still producing broods within all three exposures. Across all NDL males introduced to the hybrid tank, Trio 6 NDL was the only male to sire offspring, see Appendix 4. ALC males only fathered one offspring across the three hybrid exposures.

The genetic results observed in Figure 6 and Appendix 4 suggest that females showed a significantly higher preference for conspecifics. The LAT male showed the most heterospecific paternity, with NDL males having the lowest paternity in heterospecific tanks. The behaviour presented by each male was observed to allow inference of how each male responded to each female species.

Behavioural Analysis:

To observe the behavioural responses of males on female species the heterospecific and conspecific data points were plotted together, allowing for comparison of behaviours. This was plotted against the relative male size (%) within their trio (ALC+LAT+NDL/100). All automatic 0 data points, such as aggression towards ALC by an ALC male were removed. Allowing for a clearer analysis of viable data. All ALC males were largest in relative size (%) to the other male species within their trio, as can be observed from Figure 6 and 7, and in SL irrespective of their Trio, see Appendix 3.

Aggression:

Aggression was presented by most males across all tanks. Within each tank, hierarchies were present, with conspecifics being the more dominant within some Trios. ALC were the more aggressive and larger species. Figure 7 indicates the overall aggression across all four tanks. There does not appear to be a relationship between size and increased aggression. Males which were in conspecific tanks did not have higher frequencies in contrast to individuals housed with heterospecifics. Only one LAT male had a higher frequency of aggression when housed with conspecifics, all other male species showed no preference towards conspecifics, see Figure 7.

Courtship:

The courtship presented by each species was higher when housed with conspecifics, supporting the results in Figure 8. The highest frequency of

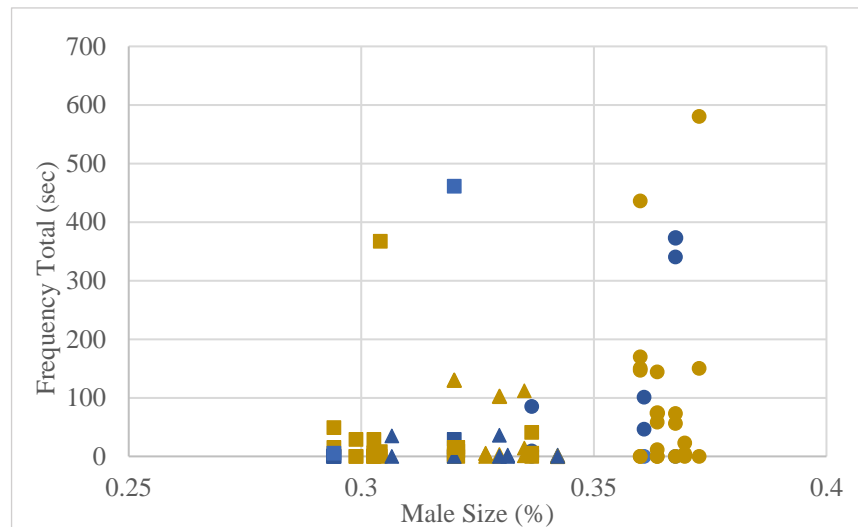


Figure 7. Graph showing the total frequency of aggression against male size in relation to trio males (%). The aggression observed is distinguished between whether the male was in a same species (blue) or different species (yellow) tank. *A. alcalica* (●) tended to be the largest in relative to the other males, followed by *A. ndalalani* (▲), and *A. latilabris* (■). There is no apparent pattern between relative male trio size and increased frequency. Males did not directly show an increased frequency of aggression when a same species tank.

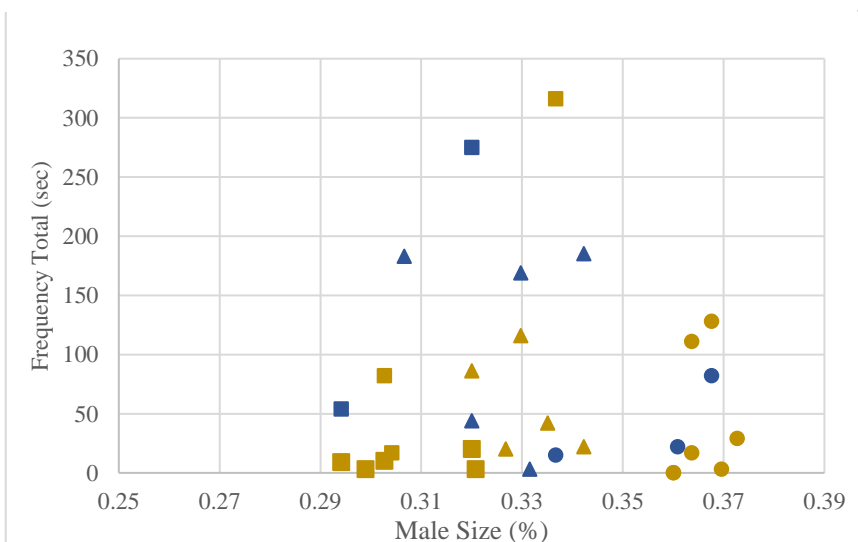


Figure 8. Overall frequency of courtship presented by all male Trios across the three species tanks. *A. ndalalani* (▲) showed the highest frequency of aggression when with their same species (blue). The largest males in relative size to their Trio males, did not show high courtship. *A. alcalica* (●) showed the highest frequency of courtship when in a different species tank (yellow). *A. latilabris* (■) showed highest courtship with their same species females.

courtship was expressed by a LAT male in a heterospecific tank, which as observed in Figure 8 was highest in relative size in contrast to other LAT males but was not the largest male. The paternity results observed in Appendix 4, indicated that the Trio 3 LAT male with its conspecifics successfully mated and produced the most offspring. In contrast, the higher courtship displays by the Trio 4 male observed in Appendix 4 and Appendix 1 had some possible successful offspring but cannot be confirmed due to allele similarity between the mother and males within their Trio. The NDL males showed higher frequencies of courtship across most Trios. The ALC males expressed higher courtship with heterospecifics. Both ALC and NDL, were not bright in colouration, as indicated in Appendix 2. As observed in Figure 6, the conspecific males sired the most offspring within their respective tank.

Territoriality:

The frequency of territoriality that each species presented varied depending on whether males were housed with conspecifics. As can be observed in Figure 9 LAT and NDL males showed higher rates of territoriality when with their same species females. The ALC male did not show a higher frequency of territoriality with conspecifics. The higher the territoriality did not

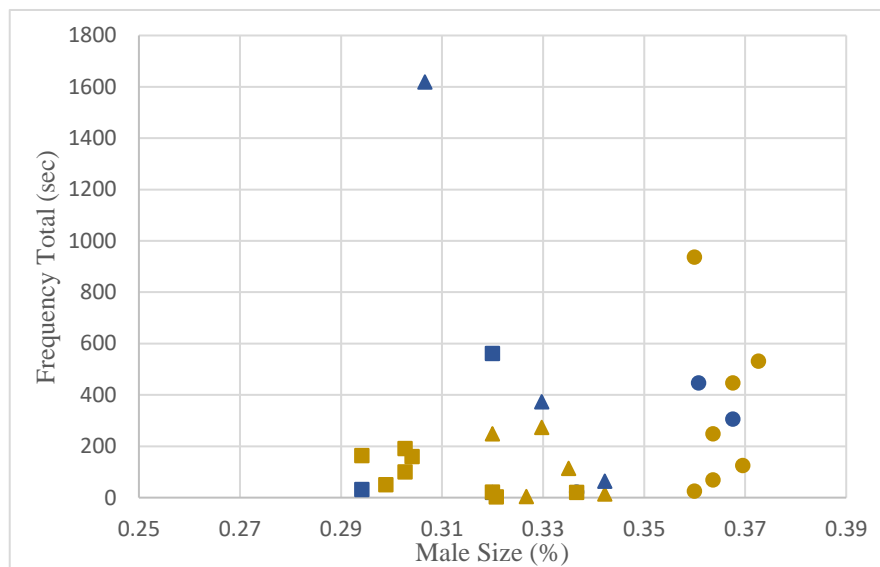


Figure 9. Frequency of overall territoriality presented by all males. *A. ndalalani* (▲) and *A. latilabris* (■) showed the highest frequency of territoriality towards conspecifics (blue). The *A. alcalica* (●) highest rate of territoriality were towards heterospecific females (yellow). The highest frequency of territoriality was by a NDL male with their conspecifics.

relate to relative male size within their trio, with smaller males showing higher frequencies of territoriality. As can be observed in Figure 9, the males which showed the highest frequency of territoriality were bright in colouration. However, across all species, the presence of high territoriality towards conspecifics was not present, with ALC males showing higher territoriality in heterospecific tanks. The highest frequency of territoriality was by the Trio 5 NDL male, this male fathered all offspring analysed, see Appendix 4.

As indicated in Table 3, each species was not introduced to all aquaria. Within each tank, the males were introduced to conspecific vs heterospecific, heterospecific vs heterospecific, conspecific vs hybrid

and hybrid vs conspecific. To further observe the variation in behaviour between species, the behaviours were compared within the specified groups.

Conspecific vs Heterospecific:

Males which were introduced to their conspecific and heterospecific tank showed some variations in behaviour between treatments. The same relationship can be observed in the paternity results, with males who fathered the most broods with their conspecifics fertilising no eggs in heterospecific tanks. Trio 1 ALC, was introduced to ALC and NDL tanks as indicated in Table 4.

Figures 7-10 show the overall frequency of behaviours each male presented during exposure. The Mann-Whitney U test indicated that there was a significant difference in CB ($U=17.5, p<0.05$), and T ($U=p<0.05$). This data suggests the Trio 1 ALC male showed a higher frequency of courtship and territoriality within the NDL tank in contrast to their conspecific tank. The paternity results in Figure 6 indicate that

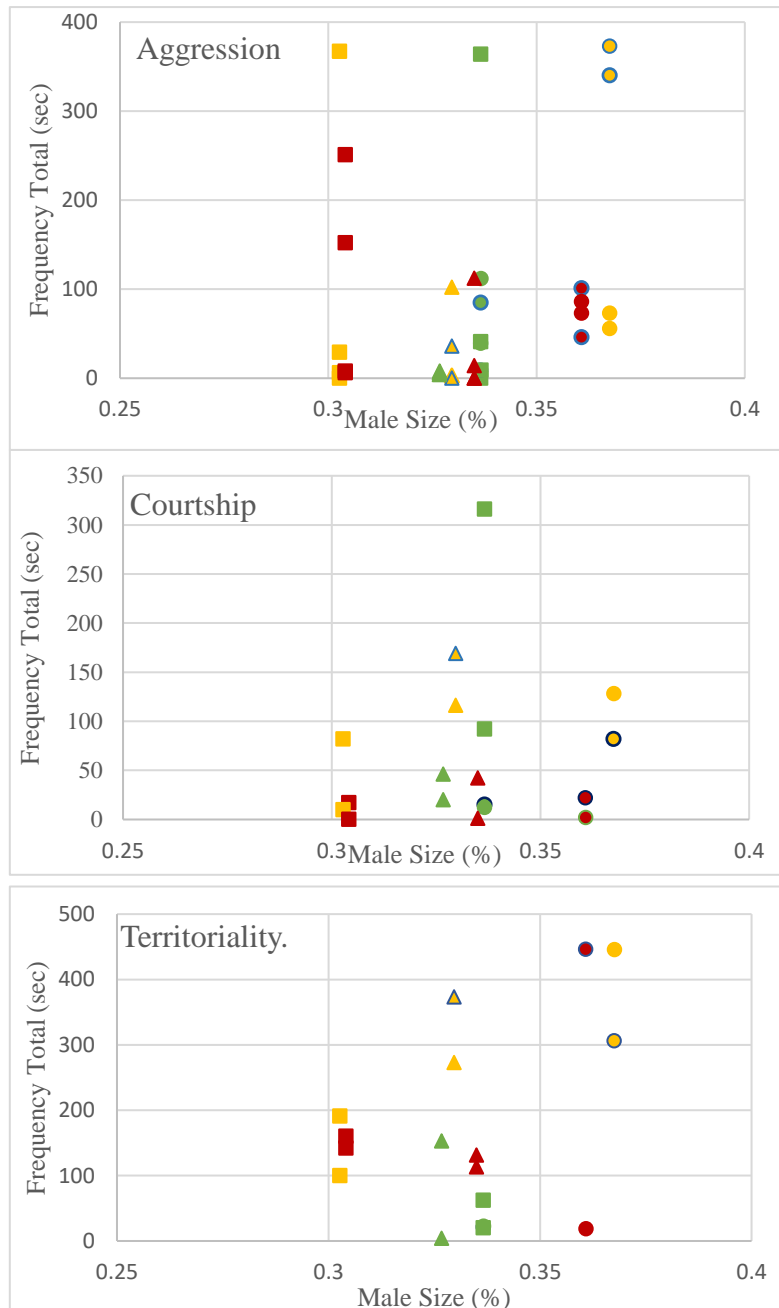


Figure 10. Frequency of aggression, courtship, territoriality presented by all Trio 1 (yellow), Trio 4 (Green), and Trio 6 (red) males. As can be observed, there does not appear to be a relationship between male species and higher behaviour frequencies, with heterospecific males showing higher courtship, territoriality, and aggression. Within each Trio there was a variation in behaviour, the males in conspecific tanks are indicated with the blue border. NDL (\blacktriangle) had higher courtship and territoriality in their conspecific tank. LAT (\blacksquare) showed high frequencies of aggression and courtship in heterospecific and hybrid tanks. The ALC (\bullet) males were largest in size, with high territoriality and aggression in their conspecific tank.

the ALC male showed assortative mating within their conspecific tank. In contrast within the female NDL tank, the Trio 1 male showed some disassortative mating, successfully copulating with a heterospecific female, but fathering less offspring than when with its conspecifics.

The Trio 1 NDL male showed a significant difference in AGL, ($U=21$, $p<0.001$) with higher aggression within its conspecific tank. The genetic results indicate assortative mating within its conspecifics, see Figure 6. There is also a presence of disassortative mating, with ALC successfully siring a high number of offspring. Figure 10 observed NDL males showing higher frequencies towards conspecifics in courtship and territoriality, although not significantly different between exposed tanks.

As indicated in Table 4, Trios 2 and 3 were introduced to the LAT and NDL female tanks. Trio 3 LAT showed a higher frequency of aggression towards NDL within their conspecific tank ($U=26$, $p<0.05$) see Figure 10. As observed in Figure 11 the Trio 3 LAT male showed a higher frequency of courtship and territoriality within their conspecific tank. However,

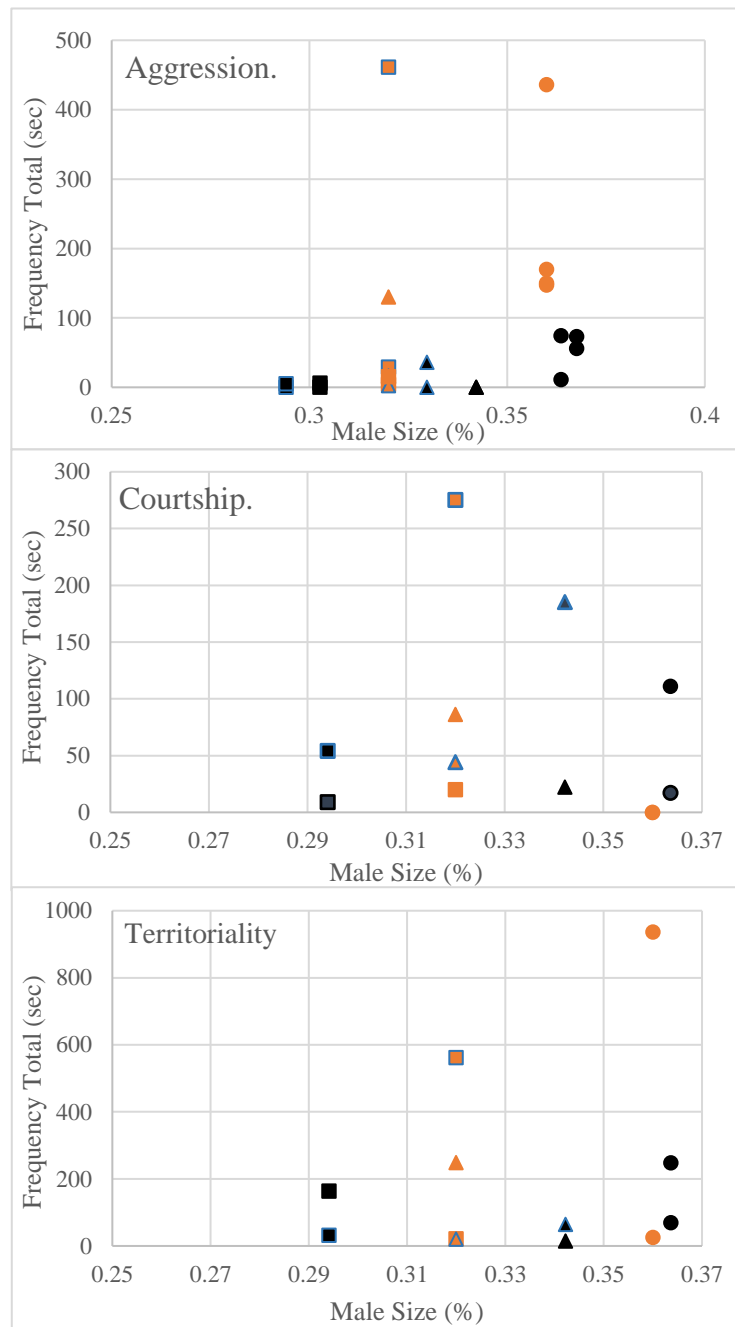


Figure 11. Frequency of aggression, courtship, and territoriality presented by Trio 2 (black), and Trio 3 (Orange). The Trio 3 LAT (■) male showed significantly higher aggression, courtship and territoriality when housed with their conspecifics indicated with the blue border. The Trio 2 NDL (▲) showed the same pattern of behaviour. This was not observed in The Trio 3 NDL male, with higher behavioural frequencies towards heterospecifics across all three behaviours. The ALC (●) male was not introduced to a conspecific tank, with Trio 3 showing some high frequencies of aggression and territoriality towards heterospecifics.

the Whitney U test indicates that there appears to be no significant difference between female tanks ($p>0.05$). The sum of frequencies, suggests that there does appear to be a higher rate of courtship presented by the Trio LAT 2 male with their conspecifics, see Figure 11. The non-parametric Whitney-U test indicates that there is no significant difference across all behaviours for the Trio 2 LAT male ($p>0.05$). The genetic results observed assortative mating was present across both Trios within their conspecific tank. ALC had successful copulations within LAT broods in the Soda 2 tank, see Appendix 4.

Figure 11 observes the frequency of behaviours presented by both Trio 2 and Trio 3 NDL males. Trio 2 NDL male showed a significantly higher frequency of CB within their conspecific tank ($U=5.5$, $p<0.01$), observed in Figure 10. Trio 3 NDL had significantly higher territoriality with their heterospecific females ($U=34$, $p<0.05$). Figure 11 observed the Trio 3 NDL showing higher behavioural frequencies within their heterospecific tank. The genetic results suggest that assortative mating is present

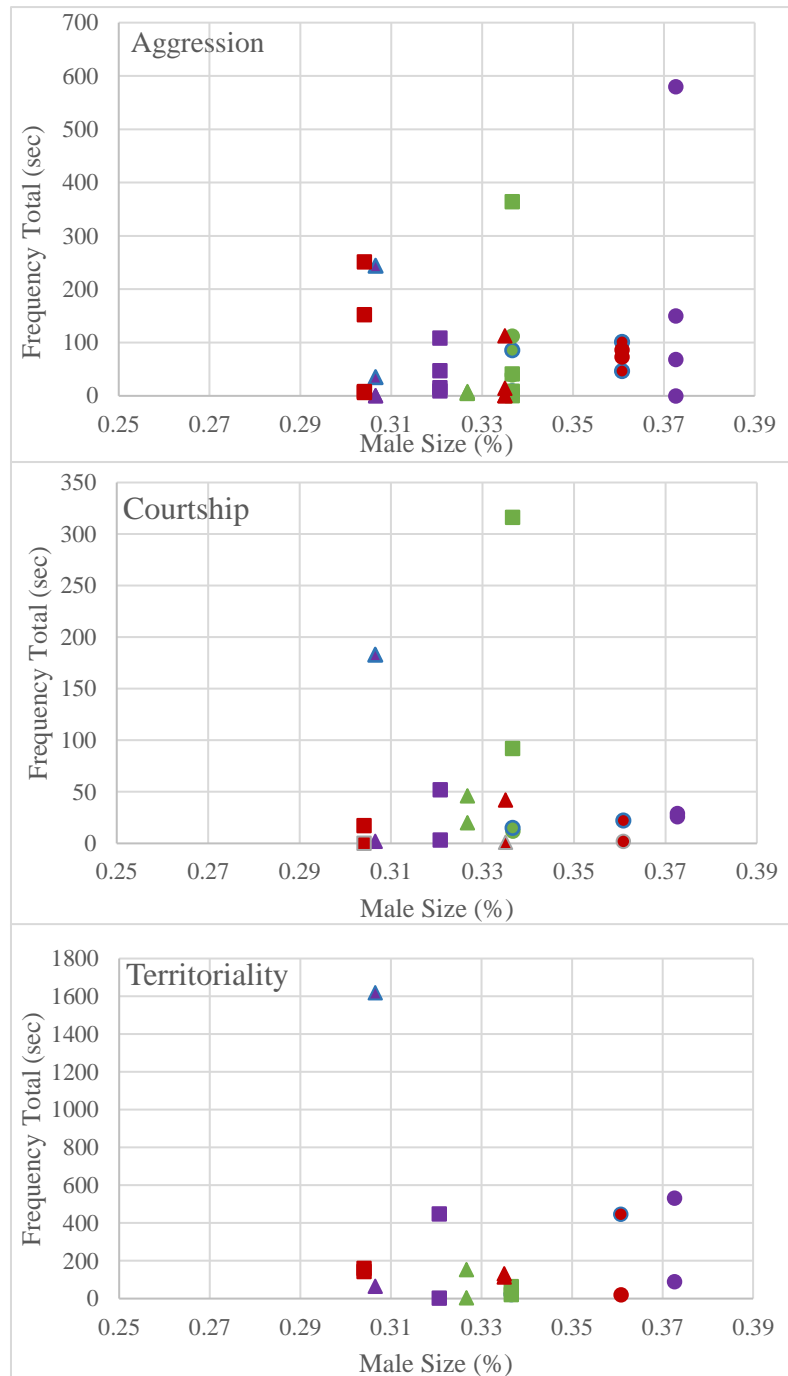


Figure 12. Frequency of aggression, courtship, and territoriality presented by Trios 4 (green), 5 (purple), and 6 (red). As can be observed, The Trio 5 NDL (\blacktriangle) showed significantly higher frequencies of courtship, aggression, and territoriality when with conspecifics, indicated by the blue border. A similar relationship can be observed with the Trio 6 ALC male (\bullet). Irrespective of their smaller relative size, the NDL and LAT (\blacksquare) males showed higher or similar behavioural frequencies to the larger ALC males. The Trio 4 LAT male had the highest frequency of courtship.

across both Trios. LAT males in both Trios fathered offspring within NDL broods in the NDL tank, see Appendix 1 and 4.

Heterospecific vs Heterospecific:

The unbalanced design introduced cases where some Trios were not introduced to all female tanks. The Trio 1 LAT, 2 ALC and 3 ALC were only introduced to heterospecific females. Neither of these males fathered broods with NDL females.

The Trio 1 LAT male showed high frequencies of courtship within Tank 1, see Figure 10. However, the Mann Whitney U test did not indicate a significant difference in behaviours between ALC and NDL females. The genetic results are insufficient to identify the paternity of some offspring due to allele similarity. However, LAT may have fathered some broods within the NDL tank, see Appendix 1, while also fathering an offspring within an ALC brood, see Appendix 4. Although non-significant, the Trio 1 LAT male did show a high frequency of aggression within the ALC female tank towards NDL, see Figure 10.

The Trio 3 ALC male showed high frequencies of territoriality and aggression within their heterospecific tanks, see Figure 11. Trio 2 and 3 ALC males had high frequencies of territoriality and aggression towards NDL. The Trio 2 ALC male had significantly higher rates of aggression towards NDL ($U=7$, $p<0.01$) within the NDL female tank. Trio 2 and Trio 3 ALC males showed significant rates of territoriality. The highest rates of territoriality by these two males were towards different female tanks, with Trio 2 showing more territoriality with NDL females ($U=17$, $p<0.05$), while the Trio 3 male had higher territoriality with LAT females ($U=23.5$, $p<0.01$). The genetic results indicate that within the offspring analysed ($N=5$) Trio 2 and 3 ALC fathered singular offspring within LAT broods. Neither Trios had any successful offspring with NDL females, see Table 3.

Conspecific vs Hybrid:

Some males were introduced to their conspecific and hybrid (*A. alcalica* x *A. latilabris*) females. This consisted of Trio 4 ALC, 5 NDL and 6 ALC.

Trio 4 and Trio 6 ALC have the same spp. parent to the hybrid. The behavioural data indicated that Trio 4 ALC showed significantly higher frequencies of territoriality towards their conspecific females ($U=13.5$, $p<0.05$), see Figure 12. The genetics observed that this male fathered five of the eight offspring analysed, in contrast to not siring any offspring within the hybrid tank, see Figure 6.

The Trio 6 ALC male fathered all offspring within their conspecific tank and none in the hybrid (see Appendix 4), supported through significantly higher frequencies of courtship towards their conspecific females ($U=8$, $p<0.01$), see Figure 10. Territoriality frequencies were also high within their conspecific tank as observed in Figure 10 but were non-significant. The Trio 6 ALC male may have fathered an

offspring with a hybrid female, but due to allele similarity paternity could not be concluded, see Appendix 1.

Figure 11 shows all the Trios introduced to the Hybrid tank. The Trio 5 NDL male showed the highest frequency of behaviours across all behaviours analysed. The Mann-Whitney U test showed significantly higher frequencies of courtship ($U=13.5$, $p<0.01$), Territoriality ($U=8$, $p<0.01$), Aggression towards ALC ($U=3.5$, $p<0.001$), and Aggression towards LAT ($U=24.5$, $p<0.05$) within their conspecific tank, observed in Figure 12. The paternity results in Figure 6 indicate that across all broods analysed ($N=24$) NDL was the only father of all the offspring. In contrast, the Trio 5 NDL male did not have any successful broods within the Hybrid tank, see Appendix 4.

Heterospecific vs Hybrid:

The Trio 4 NDL and LAT were introduced to ALC and Hybrid females. LAT showed significantly higher frequencies of courtship within the ALC tank ($U=3$, $p<0.01$) observed in Figure 12. All other behaviours recorded showed no significant difference between tanks for both species. The genetic results indicate that within the ALC tank, LAT may have fathered offspring within an ALC brood. However, due to the similarity in alleles between the female and the ALC and LAT males, this cannot be confirmed, observed in Appendix 1. In contrast, within the Hybrid tank, the LAT male fathered all broods analysed. The Trio 4 NDL male did not show any significant difference in behaviours, as observed in Figure 11, this male showed low frequencies of behaviours and did not father any broods with the hybrid females. In the ALC tank, the Trio 4 NDL male may have fathered an offspring, this cannot be confirmed due to the mother not being swabbed in error, see Appendix 1.

Trio 5 ALC and LAT were exposed to the NDL and Hybrid females. The LAT male showed significantly higher frequencies of courtship ($U=29.5$, $p<0.05$), and aggression towards ALC ($U=23$, $p<0.01$) with the hybrid females. In contrast to the behavioural observations, Table 3 observed the Trio 5 LAT male fathering the most broods within Trio 5. The ALC male did not show any significant difference in behaviour across tanks. However, as can be observed in Figure 11 there is a slightly higher frequency of territoriality in the NDL tank. As indicated in Table 3 the paternity results indicate that the Trio 5 NDL male did not father any offspring in the NDL tank, while having a single successful brood in the hybrid tank.

As can be observed in Figures 10 and 12, Trio 6 was introduced to ALC and the Hybrid females. The LAT ($U=12$, $p<0.001$), and NDL ($U=7$, $p<0.001$) males had significantly higher frequencies of courtship towards ALC females. The paternity results suggest that while showing a high frequency of courtship, neither males fathered any offspring in the ALC tank, see Table 3. The NDL male also had a significantly higher frequency of aggression towards ALC males in the ALC female tank ($U=12$, $p<0.001$) observed in Figure 11. Within the hybrid tank, the NDL male fathered most broods, with the LAT male successful siring one of the offspring analysed, see Table 3.

Overall, the LAT males appeared to have the most disassortative mating, whereby across the three species tanks (excluding the hybrid tank) fathered the most broods with heterospecific females.

Discussion:

This study indicates that the three *Alcolapia spp.* mate assortatively, as can be observed from Figure 6 with genetic data observing each species fathering the most offspring with their conspecific female. There was also a variation in heterospecific paternity; LAT males fathered broods across the most exposures, while NDL sired the least with only fathering offspring in five of the 12 exposures (four of which are with conspecifics), see Appendix 4. In relation to the behavioural data, each male tended to show the highest frequency when in their conspecific tank in contrast to heterospecifics. However, this behaviour was not present across all males. When comparing Trios which were introduced to two conspecific tanks (e.g. ALC and LAT males within the same Trio being introduced to Tank 1 and Tank 2, see Appendix 4), each male produced the most offspring with their conspecifics. The unbalanced design prevents this comparison across all males, varying the level of observations between exposures, see Appendix 4.

Species Dependent Behavioural Response:

The frequency of behaviour presented by each male varied across exposures. It can be observed from Figures 10-12, that between the two female species tank each Trio was introduced to (see Table 3), conspecific males showed the higher rates of courtship and territoriality, fathering the most broods within that exposure. This cannot be observed across all males, such as the Trio 1 ALC, Trio 3 NDL and Trio 4 ALC having lower frequencies towards conspecifics, but still siring the most broods, see Figures 10 and 11, and Appendix 4. This may suggest the possible presence of secondary factors in assortative mating. The process by which species can identify conspecifics has been widely discussed. Summers *et al.* (1999) observed the presence of colour morph assortative mate choice present in the strawberry poison frog, *Dendrobates pumilia*. Whereby, females show assortative mating only under white light, with morph calls not influencing mate choice (Summers *et al.*, 1999). However, Knight and Turner (2004) state that visual cues alone are not sufficient to distinguish between phenotypically similar species such as in the cichlid *Pseudotropheus* genus (Knight and Turner, 2004). The backup cue hypothesis suggests that even when a female preference is present, such as male size, females can distinguish between sympatric heterospecific males which show a stronger preferred trait by using chemical cues, as observed in *Xiphophorus pygmaeus* and *X. cortezi* (Hankison and Morris, 2003). *Alcolapia spp.* are morphologically divergent sympatric species, suggesting that visual cues may be used in species recognition. *Tilapia* species in Lake Ejagham, Cameroon showed strong assortative mating towards colour, size, diet, and head depth (Martin, 2013).

Male Size:

The relative size of each male showed no significant difference in the frequency of courtship, aggression, or territoriality. ALC males were the largest overall across all Trios. As can be observed in Figures 7-9, smaller males within their Trio, showed higher frequencies of courtship, aggression and territoriality than larger males in their conspecific tank. The choice for smaller males may support the “back-up signal” hypothesis stated prior by Hankison and Morris (2003). Which may suggest further cues involved, other than male size. The drivers of trait selection are still unknown for *Alcolapia*. The specialised feeding morphology, colouration, and differing microhabitats (Seegers and Tichy, 1999) may be selective traits. As observed in Figure 6, conspecifics had more successful broods, irrespective of male size.

Male Colouration:

Colour variation allows for species recognition. *Alcolapia* colouration is significantly different between the three species (see Figure 2), with intensity differing between individual males (see Appendix 2); ALC males have a yellow head and chest, LAT with a blue/grey dorsal and pink caudal fin, NDL which has a orange head, yellow chest and blue dorsal. Appendix 2 indicate the colour intensity of all males. The Trio 5 NDL and LAT expressed the highest frequencies of aggression, courtship, and territoriality in the exposures in which they fathered most or all broods, see Figure 12. As observed in Appendix 2, the Trio 5 NDL male was brightest in mate colour in contrast to all other NDL males. However, the Trio 5 LAT male was dull in colour. Male colour intensity can be an indicator of male health. The colouration of male ornaments in red grouse, *Lagopus lagopus scoticus* may be used as an indicator of male immuno-competence, and overall male quality. Variation in colour between sympatric species can suggest niche adaptations. Microhabitat variations and background colour can allow for differences in contrast to colouration (Gray and McKinnon, 2007). The colour variation in *Alcolapia* may be useful in wild populations if there is a variation in visual quality. The presence of assortative mating may suggest that visual cues such as colour may be involved whereby females show a preference towards conspecific colour morphs. Species response to conspecific recognition can vary between species and sexes. Kozak *et al.*, (2009) observed that in the three-spined sticklebacks, both sexes can recognise conspecifics, this may be through the size difference between the benthic and limnetic species, but only females are selective. As can be observed in Figure 8, courtship was presented by all males towards all female species, suggesting females being responsible for sexual isolation. This study could be manipulated to observe whether assortative mating is still observed when male mating colour cannot be seen, allowing for further investigations into whether females choose based on male colour. On occasions where males were not bright in colour (see Appendix 2), the mating still appeared to be assortative (see Appendix 4), suggesting colour intensity alone does not drive mate choice. Further

research could also be made on whether intra-specific male colouration affects mate choice, with brighter males siring more offspring.

Interspecific Aggression:

Figure 6 observes the overall aggression presented by all males. It can be observed that irrespective of female species, ALC and LAT males show the highest frequency of aggression. As observed in Figures 11 and 12, the Trio 3 NDL and Trio 5 NDL showed a significantly higher frequency of aggression within their conspecific tanks in contrast to heterospecific or hybrids. Grether *et al.* (2013) suggest that when sympatric species express a difference in phenotypic traits this is accompanied by a decrease in heterospecific aggression. The presence of heterospecific aggression may be a result of other factors such as territorial margins or food resources. Garcia and Arroyo (2002) observed that between sympatric hen harrier, *Circus cyaneus* and *C. pygargus* males show higher aggression towards conspecifics during mating season, with aggression corresponding to nest defence. This mate choice tank set up lead to the presence of interspecific male-male aggression. As stated in Figure 3 a structured environment was used, whereby, sand, rocks, plant pots and other fixtures were placed in the tank. According to Kelley *et al.* (2006), captive-bred butterfly splitfins (*Ameba splendens*) present an increased amount of aggression in structured environments in comparison to bare tanks when kept in high densities. Shelters have also been observed to increase the amount of aggression in wild-caught fish (Mikheev *et al.*, 2005). The shelters, in this case, the plant pot, may increase the territoriality of males, which may represent the aggression observed in this study. A decrease in aggression was observed in another set up by a PhD student from York University. This was a large open tank (4x 400l tanks), where multiple males of all species were introduced to a variation in female species. This open tank layout allows for a better imitation of the natural habitat and may allow for a decrease in interspecific aggression.

Male-male aggression was also observed between the same male species. The clear sides of the tanks allowed for between tank interactions. This may have caused distraction between males. This data was not included in the results. Males also reacted to reflections. To overcome this, black dividers would have blocked some reflection and prevented between tank interactions. If using the tank set up stated previously by the PhD student, aggression may shift towards the same species or different species males. As stated by Balakrishnan and Sorenson (2006), Indigobirds, *Vidua spp*, show higher rates of aggression towards their same species. Further analysis taking into consideration intra-specific interaction may allow inference of whether males would show higher conspecific competition in contrast to their heterospecific males. However, as indicated in Figure 6 and Appendix 4, the lack of a relationship between successful broods and female species may suggest that the more dominant male does not directly influence mate choice.

Disassortative mating:

There was a presence of disassortative mating across the three species tanks. The LAT males showed the highest proportion of broods with heterospecific females; some of the broods being within conspecific male broods, see Figure 6 and Appendix 1. This may indicate the presence of sneaky mating, where males mate with females which have already been chosen by another, more dominant or territorial male (Magalhaes *et al.*, 2017). Magalhaes *et al.* (2017) suggest that sneaky mating can arise in populations with high competition. Some species such as the mouthbrooding cichlid *Ophthalmotilapia ventralis*, use territorial bowers produced by other males to court females, faking ownership (Haesler *et al.*, 2009). Sneaky mating increases male reproductive opportunity but decreases the opportunity of sexual selection towards male traits (Haesler *et al.*, 2009; Singer *et al.*, 2006; Jones *et al.*, 2001). However, the presence of sneaky copulation within this study design does not indicate whether this would be observed if all female species were present. Hebets (2003) observed that in the wolf spider, *Schizocosa uetzi* females will choose to mate with a familiar phenotype. Verzijden and Cate (2007) observed that young female Lake Victorian cichlids will imprint on mother sexual preferences, influencing reproductive isolation and assortative mating. Singh *et al.* (2020) state that in the free-living flatworm, *Macrostomum lignano* and *M. janickei*, although morphologically divergent in male reproductive organ, hybridisation is present. However, there is a higher rate of conspecific pairings in *M. lignano* due to a higher reproductive rate (Singh *et al.*, 2020), suggesting that the assortative mating observed may also be influenced by factors such as fecundity.

The males who showed higher rates of territoriality also had higher frequencies of aggression, as can be observed in Figures 10-12. *Alcolapia* expressed territoriality using flowerpots and bowers. The identification of territoriality was based on the duration spent at these structures. The ownership is gained by some cichlid species by increased aggressive behaviour (Taborsky *et al.*, 2009). The Trio 5 NDL male showed the highest frequency of aggression and courtship alongside high frequencies of territoriality; this male was the only male species in Trio 5 to successfully father offspring within the NDL female tank, see Appendix 4. Taborsky *et al.* (2009) observed that the males which were larger tended to win territoriality. The larger ALC male within this Trio presented high rates of aggression towards this NDL male, but lower rates of territoriality and courtship, contradicting the observation by Taborsky *et al.* (2009). These observations were in the NDL female tank, in contrast to the hybrid tank, there are lower frequencies of all the three behaviours and no successful broods, see Appendix 4 and Figure 12. This observation of higher frequencies of courtship in conspecific males, irrespective of territoriality, size and aggression, may suggest some possible mate choice based on other phenotypic or behavioural factors. Territorial Indigobirds, *Vidua spp.* show higher aggression in response to conspecific songs (Balakrishnan and Sorenson, 2006). The presence of heterospecific males does to instigate a competitive response in Indigobirds due to assortative mating towards song removing mating

competition, with aggression only being presented towards conspecifics males (Balakrishnan and Sorenson, 2006).

The territoriality of a species may also influence sneaky copulation (Magalhaes *et al.*, 2017; Haesler *et al.*, 2009). *Nyassachromis cf. microcephalus* differ in the presence of territorial behaviours, with territorial males having a higher reproductive success (Magalhaes *et al.*, 2017). However, less dominant, and non-territorial males benefit from sneaky copulation, through a higher probability of fathering offspring (Magalhaes *et al.*, 2017). This study observed successful mates in males which showed significant frequencies in territoriality, in conspecific and some heterospecific tanks, see Figure 10-12. The Trio 3 ALC male showed significant frequencies of territoriality, and successfully fathered an offspring within a LAT brood when housed with LAT females, as can be observed in Table 3. The Trio 3 LAT male showed higher frequencies of territoriality within their conspecific tank. In two sympatric *Laterallus* crake species, their vocal signals have diverged, but still present interspecific territoriality (Depino and Areta, 2020). Depino and Areta (2020) suggest that this is a result of aggression towards heterospecifics.

Heterospecific behaviour:

The unbalanced design of this study resulted in heterospecific pairings with males not being introduced to conspecific tanks. As observed in Figure 6, there were some disassortative mating by these males. Both ALC males showed a significant difference in territoriality between the tanks they were exposed to, suggesting no relationship between species. These males were not introduced to conspecific tanks. Female field crickets, *Gryllus rubens* and *G. texensis* mate choice differ relating to previous experiences with conspecific and heterospecific males (Izzo and Gray, 2011), suggesting that experience with male species may influence behaviour and mate choice.

Hybrid mate choice:

It would have been assumed that ALC and LAT males would have shown higher frequencies of courtship and dominance within the *A. alcalica* x *A. latilabris* hybrid tank through sharing parental species. As observed in Table 3, some males were only introduced to heterospecific and hybrids. The Trio 5 ALC male was introduced to the hybrid and heterospecific tank, as observed in Figure 12, this male showed higher or similar frequencies of aggression, territoriality and courtship towards heterospecifics. In contrast, the Trio 5 LAT male showed a significantly higher frequency of these behaviours towards the hybrid females. Figure 6 indicates that the LAT males fathered the most broods across two of the three trials. No LAT males were introduced to their conspecifics and the hybrids preventing a comparison in behaviours. The addition of observations with conspecific LAT tanks would suggest whether this male expresses a similar or higher frequency of behaviour and paternity with its same species female. As stated, other cues may be involved in species recognition. The hybrid female *Rana esculenta* (*R. lessonae* x *R. ridibunda*) choose *R. lessonae* over conspecific males (Abt and Reyer,

1993). Abt and Reyer (1993) observed that hybrid female *Rana esculenta* only produce *R. ridibunda* eggs and requiring *R. lessonae* mates to produce viable offspring. Svensson *et al.* (2017) investigated the inheritance of mate preference in two sympatric Lake Victoria cichlids species. Hybrids of these sympatric species showed the same mate choice preference as their non-hybrid parental species towards dorsal colour (Svensson *et al.*, 2017). The presence of hybridisation can impact the genetic diversity of a species (Vilà *et al.*, 2000; Perry *et al.*, 2002). This may suggest that understanding the presence of hybridisation within these species may be important in species stability, whereby, extensive hybridisation can affect the integrity of endangered species (Hailer and Leonard, 2008)

Environmental and energetic influences on mate choice:

Alcolapia has been observed to occupy varying microhabitats in Lake Natron, accompanied by specialised jaw morphology across the three *Alcolapia spp.*, see Figure 2. Sympatric speciation may influence microhabitat adaptation and divergences in diet. Variations in diet and microhabitats can introduce weak assortative mating but are insufficient in explaining assortative mating in threespine stickleback, *Gasterosteus aculeatus* (Snowberg and Bolnick, 2012). Suggesting that mate preferences must be present to introduce diet-assortative mating (Snowberg and Bolnick, 2012). Other studies have also observed a strong assortative mating towards diet (Martin, 2013) as an indirect influence of male size. which may be indicative of a variation in diet. The provision of the same diet and controlled habitat within this study removes dietary influences on the assortative mating observed.

Alcolapia are mouthbrooding species, showing no parental care after fry have been released (Seehausen and Van Alphen, 1998). Maina *et al.* (2019) observed a male to female 2:1 sex ratio for *Alcolapia grahmi*, excluding samples taken from ambient populations. This sex ratio can influence mate choice. Kvarnemo *et al.* (1995) observed a variation in behaviours dependent on sex ratios in the sand goby, *Pomatoschistus minutus*. When females were more abundant, females showed increased female-female interactions, with an increased number of males spawning. Cardinalfish *Apogon notatus* males are higher in abundance and have a reversed mating strategy (Okuda *et al.*, 2003), the males provide the mouthbrooding and their high abundance introduced sexual selection by the territorial females (Okuda *et al.*, 2003). Male *Alcolapia* presented territoriality, while females mouthbrood. The possible higher abundance of males and energetic output of mouthbrooding would suggest that the females were the choosy sex. However, other factors such as territoriality and food resources may allow for male choosiness. Byrne and Rice (2006) observed *Drosophila melanogaster* males with low resources showing increased choosiness to mate with larger more fecund females. Female fecundity is a driver of male choosiness (Bonduriansky, 2001). From the presence of male courtship behaviour in heterospecific tanks and assortative mating in Table 3, it would be suggested that females appear to be the choosy sex.

Male mate choice is variable within species (Edward and Chapman, 2011). Factors such as male resource and energetic investment can promote male mate choice, including events where male parental care is low (Edward and Chapman, 2011). Variation in female quality can promote male mate choice (Edward and Chapman, 2011). Female size can be an indicator of mating quality, through increased egg production, observed in Haplochromine cichlids, *Astatotilapia flavijosephi* (Werner and Lotem, 2003). In the funnel web spider, *Agelenopsi aperta*, males do not choose mates based on female size but use the amount of pheromone emitted (Reichert and Singer, 1995). Female size may have also been a factor in courtship frequency. Although invasive, the collection of female size may allow inference of its influence on male courtship. Further research into female size and male courtship may be an indicator of whether female size influences *Alcolapia* mate choice. Fewer samples of *A. latilabris* broods were collected. However, this may be a result of jaw morphology and less pronounced mouthbrooding. Further understanding of species fecundity may be an indicator of species stability and mate choice.

Limitations:

This study was faced with many complications. As stated, all male trios were not exposed to all treatments. This prevented the comparison of interspecific interactions. Limiting the understanding of the variation of male behaviour and paternity across species and decreasing the power of results observed. The tank set up is likely to have presented aggression due to lack of space and variation of tank density due to removal of brooding females. This could have been avoided through an increase in female numbers or rotation of females. The sex of a female could not be confirmed before the introduction, due to the method of stock housing. Multiple juvenile males were removed from the treatment tanks. It was observed in stock tanks that when larger males are removed the smaller males would show mate colouration. Before initiating the study females could be housed without adult males to allow any juvenile males to develop breeding dress and be removed. The video recordings were only analysed by one individual aware of the design set up, this could have introduced observer bias. In further studies the use of another individual to further analyse the videos would remove any possible bias in results, while also interpreting the results as a blind observer, removing further bias.

There were limitations in the genetic data collection. The initial process by which a negative control was used only allowed for observation of whether there is possible contamination. The use of a positive control would allow for a definite understanding of whether across the PCR process there are no errors, indicating whether the desired result has been achieved. The lack of previous background on this species caused limitation on the selected primer, whereby, multiple primers were tested at varying temperatures to observed optimum amplification during the PCR process. These species are closely-related preventing the use of DNA barcoding on the mtDNA, to provide a positive control and serve as identification between conspecifics. With additional assistance from UCL and German collaborators

with York University, the samples were successfully sequenced with assistance from a PhD student from York University.

Five primers were initially used to assess the paternity, after processing only four were successful. The variability between primers still allowed for paternity interpretation. However, on some samples, the similarity between males was too high to conclude paternity, see Appendix 1. If time restrictions were not present a fifth primer would have assisted in distinguishing between these individuals.

Conclusion:

A high level of assortative mating was recorded. Conspecific males generally showed more courtship and aggressive behaviour, but heterospecific males generally did attempt to court females. Some disassortative mating occurred, possibly as a result of sneaky mating. The presence of assortative mating, increased courtship and territoriality towards conspecifics, suggest the females are the choosier sex. The selected traits are still unknown. The variation in phenotypic traits, such as jaw morphology and colouration may allow for species recognition by females. The variation in male size and colour intensity did not appear to influence mate choice, with females choosing to mate with smaller less territorial conspecific males. Territoriality was high in males which showed high courtship behaviour. The hybrid females appeared to mate more with LAT males. It was also observed that males would use differing structures for territoriality, with LAT creating bowers, while ALC males tended to use structures such as flowerpots. This may suggest that the possible microhabitat partitioning in *Alcolapia* may support assortative mate choice. This study allowed for a preliminary inference in mate preferences in the sympatric *Alcolapia* spp. Refinements on study design, such as increased tank size and stock density, may allow for this study to be performed following a balanced design, with each Trio being exposed to each female species, providing a better overview of differences in male behaviours in contrast to conspecifics. Further research using an imitation of how these species coexist in Lake Natron, by using a mixed female and male species would allow for a better observation on how behaviours are expressed in Lake Natron.

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APPENDIX1:

| ALC | | | | Blue | | | | Red | | | | Black | | | | Green | | | | Paternity |
|---------|-----------|-------|--------|----------|--------|----------|--------|----------|--------|----------|-----|----------|-----|----------|-------|----------|-------|----------|-----|-----------|
| | Offspring | | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | |
| Trio | Sample | Plate | Well | Size | int | Size | int | Size | int | Size | int | Size | Int | Size | int | Size | int | Size | int | |
| 1 | 1.1.1.1 | 6 | B2 | 180.96 | 181 | 187.22 | 187 | 247.78 | 248 | 247.78 | 248 | 290.76 | 290 | 298.13 | 298 | 345.59 | 345 | 345.59 | 345 | 1A |
| | 1.1.1.2 | 6 | C2 | 181 | 181 | 187.27 | 187 | 248 | 248 | 248 | 248 | 290.74 | 290 | 298.22 | 298 | 341.44 | 341 | 341.44 | 341 | 1A |
| | 1.1.2.1 | 6 | F3 | 181.03 | 181 | 199.73 | 199 | 248.04 | 248 | 248.04 | 248 | 298.12 | 298 | 302.22 | 302 | 333.01 | 333 | 345.68 | 345 | 1A |
| | 1.1.2.2 | 6 | G3 | 181.01 | 181 | 199.73 | 199 | 247.96 | 248 | 247.96 | 248 | 292.55 | 292 | 292.55 | 292 | 332.96 | 333 | 341.42 | 341 | 1A |
| | 1.1.2.3 | 6 | H3 | 199.82 | 199 | 199.82 | 199 | 247.99 | 248 | 247.99 | 248 | 292.58 | 292 | 302.26 | 302 | 333.08 | 333 | 345.7 | 345 | 1A |
| | 1.1.3.1 | 6 | A4 | 181 | 181 | 199.73 | 199 | 246 | 246 | 248.02 | 248 | 292.54 | 292 | 307.41 | 307 | 330.52 | 330 | 332.94 | 333 | 1A |
| | 1.1.3.2 | 6 | D4 | 199.64 | 199 | 199.64 | 199 | 245.93 | 246 | 247.95 | 248 | 292.53 | 292 | 307.41 | 307 | 330.7 | 330 | 332.85 | 333 | 1A |
| | 1.1.3.3 | 6 | B4 | 187.15 | 187 | 199.64 | 199 | 245.89 | 246 | 245.89 | 246 | 283.58 | 283 | 307.41 | 307 | 343.46 | 343 | 345.59 | 345 | 1L |
| 4 | 1.4.1.1 | 6 | A8 | 180.99 | 181 | 191.39 | 191 | 248.01 | 248 | 248.01 | 248 | 292.6 | 292 | 302.21 | 302 | 341.51 | 341 | 345.68 | 345 | 4L? |
| | 1.4.1.2 | 6 | B8 | 180.97 | 181 | 199.73 | 199 | 247.87 | 248 | 247.83 | 248 | 292.49 | 292 | 292.49 | 292 | 341.35 | 341 | 345.55 | 345 | 4A or 4L |
| | 1.4.1.3 | 6 | C8 | 180.93 | 181 | 199.73 | 199 | 248.03 | 248 | 248.03 | 248 | 298.14 | 298 | 302.19 | 302 | 341.33 | 341 | 341.33 | 341 | 4A |
| | 1.4.2.1 | 6 | B7 | 180.9 | 181 | 216.16 | 216 | 248.01 | 248 | 248.01 | 248 | 292.51 | 292 | 307.34 | 307 | 330.72 | 330 | 341.45 | 341 | 4A |
| | 1.4.2.2 | 6 | C7 | 181.01 | 181 | 199.73 | 199 | 248.07 | 248 | 248.07 | 248 | 296.42 | 296 | 298.21 | 298 | 330.62 | 330 | 341.44 | 341 | 4A |
| | 1.4.2.3 | 6 | D7 | 180.9 | 181 | 216.21 | 216 | 245.93 | 246 | 247.99 | 248 | 292.48 | 292 | 307.36 | 307 | 330.62 | 330 | 345.55 | 345 | 4A |
| | 1.4.3.1 | 6 | D10 | 180.89 | 181 | 180.89 | 181 | 247.94 | 248 | 247.94 | 248 | 294.39 | 294 | 298.1 | 298 | 332.8 | 333 | 345.47 | 345 | 4A |
| | 1.4.3.2 | 6 | B12 | 181.11 | 181 | 181.11 | 181 | 248.04 | 248 | 248.04 | 248 | 292.59 | 292 | 298.26 | 298 | 341.56 | 341 | 345.7 | 345 | 4A |
| 1.4.3.3 | 6 | F9 | 180.92 | 181 | 180.92 | 181 | 249.27 | 249 | 249.27 | 249 | 300 | 300 | 300 | 300 | 333.1 | 333 | 345.5 | 345 | 4N? | |
| 6 | 1.6.1.1 | 6 | B11 | 181.11 | 181 | 181.11 | 181 | 248.01 | 248 | 248.01 | 248 | 292.57 | 292 | 292.57 | 292 | 341.5 | 341 | 345.63 | 345 | 6A |
| | 1.6.1.2 | 6 | G12 | 181.03 | 181 | 181.03 | 181 | 248.08 | 248 | 248.08 | 248 | 298.24 | 298 | 302.19 | 302 | 341.49 | 341 | 345.58 | 345 | 6A |
| | | | | | | | | | | | | | | | | | | | | |
| | Female | | | | | | | | | | | | | | | | | | | |
| | 1.1.2 | 5 | E7 | 180.9 | 181 | 199.73 | 199 | 248.06 | 248 | 248.06 | 248 | 292.54 | 292 | 302.19 | 302 | 341.35 | 341 | 345.55 | 345 | |
| | 1.1.3 | 5 | F7 | 199.73 | 199 | 216.23 | 216 | 245.92 | 246 | 245.92 | 246 | 296.4 | 296 | 307.41 | 307 | 330.61 | 330 | 343.52 | 343 | |

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|-------------|---|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|
| 1.4.1 | 5 | G7 | 180.96 | 181 | 199.64 | 199 | 248.02 | 248 | 248.02 | 248 | 292.55 | 292 | 302.2 | 302 | 341.5 | 341 | 345.63 | 345 |
| 1.4.2 | 5 | H7 | 199.74 | 199 | 216.23 | 216 | 245.97 | 246 | 245.97 | 246 | 296.37 | 296 | 307.43 | 307 | 330.77 | 330 | 343.6 | 343 |
| 1.6.1 | 5 | A8 | 181.08 | 181 | 199.73 | 199 | 248 | 248 | 248 | 248 | 292.52 | 292 | 302.21 | 302 | 341.51 | 341 | 345.68 | 345 |
| Male | | | | | | | | | | | | | | | | | | |
| 1A | 5 | F10 | 180.95 | 181 | 199.64 | 199 | 248.04 | 248 | 248.04 | 248 | 292.53 | 292 | 298.2 | 298 | 332.86 | 333 | 332.86 | 333 |
| 1L | 5 | G10 | 187.24 | 187 | 193.5 | 193 | 245.98 | 246 | 248 | 248 | 283.61 | 283 | 290.71 | 290 | 341.4 | 341 | 345.58 | 345 |
| 1N | 5 | H10 | 191.4 | 191 | 193.46 | 193 | 245.96 | 246 | 248 | 248 | 291.11 | 290 | 305.87 | 305 | 337.3 | 337 | 341.5 | 341 |
| 4A | 5 | G11 | 180.95 | 181 | 180.95 | 181 | 248.06 | 248 | 248.06 | 248 | 292.53 | 292 | 298.22 | 298 | 341.51 | 341 | 345.68 | 345 |
| 4L | 5 | H11 | 191.5 | 191 | 193.54 | 193 | 248.03 | 248 | 250.04 | 250 | 283.55 | 283 | 292.56 | 292 | 339.47 | 339 | 345.71 | 345 |
| 4N | 5 | A12 | 191.62 | 191 | 197.74 | 197 | 248.04 | 248 | 250.11 | 250 | 296.54 | 296 | 300.27 | 300 | 333.16 | 333 | 337.46 | 337 |
| 6A | 5 | E12 | 181.02 | 181 | 181.02 | 181 | 248.05 | 248 | 248.05 | 248 | 292.57 | 292 | 298.23 | 298 | 332.98 | 333 | 341.5 | 341 |
| 6L | 5 | F12 | 191.46 | 191 | 193.53 | 193 | 248.03 | 248 | 250.06 | 250 | 286.81 | 287 | 290.71 | 290 | 339.37 | 339 | 339.37 | 339 |
| 6N | 5 | G12 | 191.45 | 191 | 193.5 | 193 | 245.94 | 246 | 248.06 | 248 | 291.18 | 290 | 300.27 | 300 | 337.32 | 337 | 341.57 | 341 |

| LAT | | | | Blue | | | | Red | | | | Black | | | | Green | | | | |
|------|-----------|-------|------|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|-----------|
| | Offspring | | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Paternity |
| Trio | Sample | Plate | Well | size | int | size | int | size | int | size | int | size | int | size | int | size | int | size | int | |
| 2 | 2.2.1.2 | 6 | D2 | 197.62 | 197 | 205.84 | 205 | 245.89 | 246 | 247.97 | 248 | 290.78 | 290 | 290.78 | 290 | 337.26 | 337 | 339.38 | 339 | LAT2B |
| | 2.2.1.3 | 6 | E2 | 180.98 | 181 | 191.55 | 191 | 248.03 | 248 | 248.03 | 248 | 283.65 | 283 | 292.67 | 292 | 332.99 | 333 | 339.37 | 339 | 2A |
| | 2.2.1.4 | 6 | F2 | 181.06 | 181 | 205.95 | 205 | 245.93 | 246 | 248.03 | 248 | 290.73 | 290 | 292.65 | 292 | 339.29 | 339 | 341.49 | 341 | LAT2B? |
| | | | | | | | | | | | | | | | | | | | | |
| 3 | 2.3.1.1 | 6 | F10 | 191.33 | 191 | 193.43 | 193 | 247.95 | 248 | 247.95 | 248 | 286.79 | 287 | 290.65 | 290 | 339.27 | 339 | 345.55 | 345 | 3L |
| | 2.3.1.2 | 6 | E12 | 187.29 | 187 | 191.51 | 191 | 245.98 | 246 | 248.01 | 248 | 283.58 | 283 | 286.85 | 287 | 339.37 | 339 | 345.63 | 345 | 3L |
| | 2.3.1.3 | 6 | F11 | 181.01 | 181 | 205.86 | 205 | 248 | 248 | 248 | 248 | 290.77 | 290 | 298.23 | 298 | 332.98 | 333 | 339.37 | 339 | 3A |
| | 2.3.2.1 | 6 | C11 | 191.38 | 191 | 193.52 | 193 | 247.98 | 248 | 250 | 250 | 283.51 | 283 | 286.84 | 287 | 339.38 | 339 | 345.58 | 345 | 3L |
| | 2.3.2.2 | 6 | H12 | 191.41 | 191 | 193.54 | 193 | 248.06 | 248 | 250.15 | 250 | 286.84 | 287 | 290.76 | 290 | 345.7 | 345 | 345.7 | 345 | 3L |
| | Female | | | | | | | | | | | | | | | | | | | |
| | 2.2.1 | 6 | C9 | 191.36 | 191 | 197.64 | 197 | 248.03 | 248 | 248.03 | 248 | 290.88 | 290 | 290.88 | 290 | 339.27 | 339 | 341.35 | 341 | |
| | 2.2.2 | 5 | B8 | 191.41 | 191 | 193.49 | 193 | 245.93 | 246 | 247.99 | 248 | 283.53 | 283 | 292.57 | 292 | 339.27 | 339 | 341.35 | 341 | |
| | 2.3.1 | 5 | C8 | 191.4 | 191 | 205.76 | 205 | 245.9 | 246 | 247.94 | 248 | 283.54 | 283 | 290.71 | 290 | 339.28 | 339 | 339.28 | 339 | |

| | | | | | | | | | | | | | | | | | | | | |
|--|-------|---|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--|
| | 2.3.2 | 5 | D8 | 191.34 | 191 | 205.81 | 205 | 247.98 | 248 | 250.07 | 250 | 283.6 | 283 | 290.76 | 290 | 339.26 | 339 | 345.56 | 345 | |
| | Male | | | | | | | | | | | | | | | | | | | |
| | 2A | 5 | A11 | 180.95 | 181 | 180.95 | 181 | 248.06 | 248 | 248.06 | 248 | 292.69 | 292 | 302.25 | 302 | 333.02 | 333 | 341.56 | 341 | |
| | 2L | 5 | B11 | 191.51 | 191 | 193.57 | 193 | 248.03 | 248 | 250.07 | 250 | 283.59 | 283 | 290.77 | 290 | 339.46 | 339 | 339.46 | 339 | |
| | 2N | 5 | C11 | 187.24 | 187 | 197.69 | 197 | 248.07 | 248 | 248.07 | 248 | 291.23 | 290 | 300.27 | 300 | 337.24 | 337 | 341.49 | 341 | |
| | LAT2B | 6 | H9 | 187.33 | 187 | 205.9 | 205 | 245.97 | 246 | 248 | 248 | 286.89 | 287 | 290.76 | 290 | 339.38 | 339 | 341.49 | 341 | |
| | 3A | 5 | D11 | 181.06 | 181 | 181.06 | 181 | 248.01 | 248 | 248.01 | 248 | 292.57 | 292 | 298.17 | 298 | 333 | 333 | 341.47 | 341 | |
| | 3L | 5 | E11 | 187.18 | 187 | 193.52 | 193 | 245.91 | 246 | 247.96 | 248 | 286.71 | 287 | 290.61 | 290 | 339.37 | 339 | 345.63 | 345 | |
| | 3N | 5 | F11 | 187.31 | 187 | 191.46 | 191 | 245.95 | 246 | 250.11 | 250 | 291.07 | 290 | 305.85 | 305 | 337.21 | 337 | 341.5 | 341 | |

| NDL | Offspring | Plate | Well | Blue | | | | Red | | | | Black | | | | Green | | | | Paternity |
|----------|-----------|----------|------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|-----------|
| | | | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | |
| Trio | Sample | Plate | Well | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Paternity |
| 1 | 3.1.1.1 | 6 | D8 | 187.26 | 187 | 191.4 | 191 | 247.94 | 248 | 247.94 | 248 | 291.05 | 290 | 305.74 | 305 | 341.28 | 341 | 341.28 | 341 | 1N |
| | 3.1.1.2 | 6 | E8 | 193.41 | 193 | 197.62 | 197 | 245.92 | 246 | 247.99 | 248 | 291.08 | 290 | 305.84 | 305 | 341.36 | 341 | 341.36 | 341 | 1L/1N |
| | 3.1.1.4 | 6 | G8 | 193.38 | 193 | 197.56 | 197 | 247.97 | 248 | 247.97 | 248 | 291.08 | 290 | 305.8 | 305 | 336.92 | 337 | 336.92 | 337 | 1N |
| | 3.1.2.1 | 6 | A10 | 191.49 | 191 | 193.55 | 193 | 245.88 | 246 | 248.01 | 248 | 290.61 | 290 | 305.84 | 305 | 341.25 | 341 | 341.25 | 341 | 1N |
| | 3.1.2.3 | 6 | G11 | 193.42 | 193 | 193.42 | 193 | 247.95 | 248 | 247.95 | 248 | 290.73 | 290 | 290.73 | 290 | 341.51 | 341 | 341.51 | 341 | 1L/1N |
| | 3.1.3.1 | 6 | G10 | 197.68 | 197 | 199.73 | 199 | 247.99 | 248 | 250.1 | 250 | 291.14 | 290 | 298.24 | 298 | 332.86 | 333 | 341.32 | 341 | 1A |
| | 3.1.3.2 | 6 | D12 | 187.31 | 187 | 199.65 | 199 | 247.98 | 248 | 247.98 | 248 | 291.14 | 290 | 298.25 | 298 | 332.97 | 333 | 341.49 | 341 | 1A |
| | 3.1.3.3 | 6 | G9 | 187.24 | 187 | 193.41 | 193 | 247.97 | 248 | 250 | 250 | 291.13 | 290 | 291.13 | 290 | 341.31 | 341 | 341.31 | 341 | 1L/1N |
| | 3.1.4.1 | 6 | E10 | 191.33 | 191 | 193.43 | 193 | 241.82 | 241 | 245.95 | 246 | 296.41 | 296 | 305.79 | 305 | 341.36 | 341 | 341.36 | 341 | 3N? |
| | 3.1.4.2 | 6 | C12 | 181.06 | 181 | 193.57 | 193 | 248.05 | 248 | 248.05 | 248 | 294.37 | 294 | 298.21 | 298 | 332.9 | 333 | 341.44 | 341 | |
| | 3.1.5.1 | 6 | H10 | 193.46 | 193 | 197.59 | 197 | 245.87 | 246 | 250.04 | 250 | 294.4 | 294 | 305.87 | 305 | 337.3 | 337 | 341.5 | 341 | 1N? |
| | 3.1.5.2 | 6 | F12 | 191.42 | 191 | 197.59 | 197 | 245.98 | 246 | 245.98 | 246 | 291.16 | 290 | 300.18 | 300 | 337.22 | 337 | 341.5 | 341 | 1L/1N |
| | 3.1.6.1 | 6 | A11 | 181.04 | 181 | 197.71 | 197 | 246.04 | 246 | 248.13 | 248 | 298.26 | 298 | 305.91 | 305 | 333.03 | 333 | 337.27 | 337 | 1A |
| | 2 | 3.2b.1.2 | 6 | F8 | 187.19 | 187 | 193.38 | 193 | 241.78 | 241 | 247.96 | 248 | 283.52 | 283 | 286.84 | 287 | 332.81 | 333 | 339.27 | 339 |
| 3.2b.2.1 | | 6 | D11 | 187.29 | 187 | 191.48 | 191 | 248.03 | 248 | 248.03 | 248 | 296.42 | 296 | 300.27 | 300 | 341.3 | 341 | 341.3 | 341 | 2N |
| 3.2b.2.2 | | 6 | H8 | 197.67 | 197 | 197.67 | 197 | 241.85 | 241 | 248.07 | 248 | 296.36 | 296 | 300.28 | 300 | 341.5 | 341 | 341.5 | 341 | 2N |
| 3 | 3.3.1.1 | 6 | E1 | 187.43 | 187 | 197.76 | 197 | 245.96 | 246 | 245.96 | 246 | 291.17 | 290 | 291.17 | 290 | 337.39 | 337 | 337.39 | 337 | 3N |

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|---|---------|---|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|-----|
| | 3.3.1.2 | 6 | F1 | 187.39 | 187 | 191.5 | 191 | 248.09 | 248 | 250.15 | 250 | 305.98 | 305 | 305.98 | 305 | 341.62 | 341 | 341.62 | 341 | |
| | 3.3.1.3 | 6 | G1 | 187.46 | 187 | 191.6 | 191 | 248.07 | 248 | 250.16 | 250 | 291.12 | 290 | 305.98 | 305 | 341.63 | 341 | 341.63 | 341 | 3N |
| | 3.3.1.4 | 6 | H1 | 187.38 | 187 | 191.6 | 191 | 246.02 | 246 | 246.02 | 246 | 291.15 | 290 | 291.15 | 290 | 337.45 | 337 | 337.45 | 337 | 3N |
| | 3.3.1.5 | 6 | A2 | 181.01 | 181 | 193.52 | 193 | 245.83 | 246 | 247.94 | 248 | 283.58 | 283 | 298.16 | 298 | 341.47 | 341 | 345.62 | 345 | 3L? |
| 5 | 3.5.0.1 | 6 | E6 | 197.58 | 197 | 197.58 | 197 | 247.98 | 248 | 250.02 | 250 | 305.75 | 305 | 305.75 | 305 | 337.12 | 337 | 337.12 | 337 | 5N |
| | 3.5.0.2 | 6 | F6 | 197.52 | 197 | 197.52 | 197 | 247.98 | 248 | 250.07 | 250 | 283.6 | 283 | 291.12 | 290 | 332.9 | 333 | 337.14 | 337 | 5N |
| | 3.5.0.3 | 6 | G6 | 187.23 | 187 | 197.65 | 197 | 247.97 | 248 | 247.97 | 248 | 283.58 | 283 | 305.8 | 305 | 337.19 | 337 | 337.19 | 337 | 5N |
| | 3.5.0.4 | 6 | H6 | 187.26 | 187 | 191.52 | 191 | 241.77 | 241 | 247.93 | 248 | 305.82 | 305 | 305.82 | 305 | 337.19 | 337 | 337.19 | 337 | 5N |
| | 3.5.0.5 | 6 | A7 | 187.25 | 187 | 197.64 | 197 | 241.9 | 241 | 248.06 | 248 | 291.08 | 290 | 305.82 | 305 | 337.28 | 337 | 341.42 | 341 | 5N |
| | 3.5.1.1 | 6 | G2 | 197.71 | 197 | 197.71 | 197 | 241.92 | 241 | 248.12 | 248 | 283.67 | 283 | 300.27 | 300 | 337.34 | 337 | 341.56 | 341 | 5N |
| | 3.5.2.1 | 6 | H2 | 187.37 | 187 | 191.6 | 191 | 241.79 | 241 | 250.05 | 250 | 283.63 | 283 | 305.96 | 305 | 337.35 | 337 | 337.35 | 337 | 5N |
| | 3.5.2.2 | 6 | A3 | 187.31 | 187 | 197.67 | 197 | 248.05 | 248 | 248.05 | 248 | 283.63 | 283 | 291.15 | 290 | 337.21 | 337 | 341.42 | 341 | 5N |
| | 3.5.2.3 | 6 | B3 | 187.28 | 187 | 191.47 | 191 | 241.83 | 241 | 250.09 | 250 | 283.57 | 283 | 291.2 | 290 | 337.08 | 337 | 337.08 | 337 | 5N |
| | 3.5.2.4 | 6 | C3 | 197.68 | 197 | 197.68 | 197 | 248.01 | 248 | 248.01 | 248 | 296.38 | 296 | 305.8 | 305 | 332.93 | 333 | 337.14 | 337 | 5N |
| | 3.5.2.5 | 6 | D3 | 191.51 | 191 | 197.63 | 197 | 241.84 | 241 | 250.11 | 250 | 305.73 | 305 | 305.73 | 305 | 332.86 | 333 | 341.36 | 341 | 5N |
| | 3.5.3.1 | 6 | E4 | 191.28 | 191 | 191.28 | 191 | 241.78 | 241 | 241.78 | 241 | 283.65 | 283 | 305.78 | 305 | 337.14 | 337 | 337.14 | 337 | 5N |
| | 3.5.3.2 | 6 | F4 | 191.45 | 191 | 191.45 | 191 | 241.85 | 241 | 248.05 | 248 | 305.8 | 305 | 305.8 | 305 | 337.17 | 337 | 337.17 | 337 | 5N |
| | 3.5.3.3 | 6 | G4 | 197.59 | 197 | 197.59 | 197 | 241.8 | 241 | 248.06 | 248 | 305.79 | 305 | 305.79 | 305 | 332.94 | 333 | 341.41 | 341 | 5N |
| | 3.5.3.4 | 6 | H4 | 197.69 | 197 | 197.69 | 197 | 241.77 | 241 | 248.04 | 248 | 283.56 | 283 | 296.37 | 296 | 337.22 | 337 | 341.5 | 341 | 5N |
| | 3.5.3.5 | 6 | A5 | 191.49 | 191 | 191.49 | 191 | 241.8 | 241 | 248.02 | 248 | 283.51 | 283 | 305.87 | 305 | 337.16 | 337 | 337.16 | 337 | 5N |
| | 3.5.4.1 | 6 | B5 | 191.49 | 191 | 193.55 | 193 | 241.86 | 241 | 250.17 | 250 | 294.44 | 294 | 305.91 | 305 | 337.22 | 337 | 341.41 | 341 | 5N |
| | 3.5.5.1 | 6 | D5 | 193.51 | 193 | 197.63 | 197 | 241.79 | 241 | 245.95 | 246 | 283.55 | 283 | 294.4 | 294 | 337.06 | 337 | 341.36 | 341 | 5N |
| | 3.5.5.2 | 6 | E5 | 193.45 | 193 | 197.64 | 197 | 241.76 | 241 | 245.89 | 246 | 283.58 | 283 | 305.8 | 305 | 337.08 | 337 | 337.08 | 337 | 5N |
| | 3.5.5.3 | 6 | F5 | 191.45 | 191 | 197.64 | 197 | 241.85 | 241 | 245.98 | 246 | 283.58 | 283 | 294.44 | 294 | 332.89 | 333 | 337.18 | 337 | 5N |
| | 3.5.5.4 | 6 | G5 | 191.5 | 191 | 193.5 | 193 | 241.81 | 241 | 248.06 | 248 | 305.78 | 305 | 305.78 | 305 | 337.18 | 337 | 337.18 | 337 | 5N |
| | 3.5.6.1 | 6 | B10 | 191.46 | 191 | 191.46 | 191 | 245.94 | 246 | 247.98 | 248 | 283.58 | 283 | 305.84 | 305 | 332.87 | 333 | 337.21 | 337 | 5N |
| | 3.5.6.2 | 6 | H11 | 191.59 | 191 | 191.59 | 191 | 245.92 | 246 | 248.03 | 248 | 305.8 | 305 | 305.8 | 305 | 337.24 | 337 | 337.24 | 337 | 5N |
| | 3.5.6.3 | 6 | B9 | 191.34 | 191 | 197.59 | 197 | 241.79 | 241 | 245.92 | 246 | 283.53 | 283 | 283.53 | 283 | 337.07 | 337 | 337.07 | 337 | 5N |
| | Female | | | | | | | | | | | | | | | | | | | |
| | 3.1.1. | 5 | E8 | 187.29 | 187 | 197.63 | 197 | 248.04 | 248 | 248.04 | 248 | 291.16 | 290 | 305.84 | 305 | 337.07 | 337 | 341.36 | 341 | |
| | 3.1.2 | 5 | F8 | 193.48 | 193 | 193.48 | 193 | 241.86 | 241 | 248.03 | 248 | 290.72 | 290 | 290.72 | 290 | 337.09 | 337 | 341.35 | 341 | |

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|--------|---|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|
| 3.1.3 | 5 | G8 | 187.23 | 187 | 197.56 | 197 | 248.03 | 248 | 250.09 | 250 | 291.17 | 290 | 305.88 | 305 | 337.2 | 337 | 341.42 | 341 |
| 3.1.5 | 5 | H8 | 187.3 | 187 | 197.67 | 197 | 245.87 | 246 | 248 | 248 | 294.4 | 294 | 300.18 | 300 | 337.2 | 337 | 341.42 | 341 |
| 3.1.6 | 5 | A9 | 193.46 | 193 | 197.68 | 197 | 245.99 | 246 | 248.03 | 248 | 294.51 | 294 | 305.93 | 305 | 337.22 | 337 | 341.5 | 341 |
| 3.2b.1 | 5 | B9 | 191.45 | 191 | 193.41 | 193 | 241.78 | 241 | 241.78 | 241 | 283.59 | 283 | 290.7 | 290 | 332.88 | 333 | 341.4 | 341 |
| 3.2b.2 | 5 | C9 | 191.45 | 191 | 197.64 | 197 | 241.81 | 241 | 248 | 248 | 296.33 | 296 | 305.82 | 305 | 337.18 | 337 | 341.44 | 341 |
| 3.3.1 | 6 | A9 | 187.25 | 187 | 197.68 | 197 | 245.95 | 246 | 245.95 | 246 | 290.01 | 290 | 290.89 | 290 | 337.22 | 337 | 337.22 | 337 |
| 3.5.1 | 5 | D9 | 187.2 | 187 | 197.65 | 197 | 245.98 | 246 | 248.03 | 248 | 294.39 | 294 | 300.28 | 300 | 337.2 | 337 | 341.42 | 341 |
| 3.5.2 | 5 | E9 | 187.28 | 187 | 197.65 | 197 | 248.01 | 248 | 250.07 | 250 | 291.05 | 290 | 305.8 | 305 | 337.18 | 337 | 341.44 | 341 |
| 3.5.3 | 5 | F9 | 191.46 | 191 | 197.62 | 197 | 241.87 | 241 | 248.1 | 248 | 296.39 | 296 | 305.84 | 305 | 337.07 | 337 | 341.36 | 341 |
| 3.5.4 | 5 | G9 | 193.5 | 193 | 197.6 | 197 | 247.98 | 248 | 250 | 250 | 294.39 | 294 | 300.18 | 300 | 337.15 | 337 | 341.41 | 341 |
| 3.5.5 | 5 | H9 | 193.5 | 193 | 197.69 | 197 | 245.94 | 246 | 247.97 | 248 | 294.44 | 294 | 305.93 | 305 | 337.32 | 337 | 341.57 | 341 |
| 3.5.6 | 5 | A10 | 191.49 | 191 | 191.49 | 191 | 241.82 | 241 | 245.9 | 246 | 283.59 | 283 | 305.87 | 305 | 333.06 | 333 | 337.3 | 337 |
| Male | | | | | | | | | | | | | | | | | | |
| 1A | 5 | F10 | 180.95 | 181 | 199.64 | 199 | 248.04 | 248 | 248.04 | 248 | 292.53 | 292 | 298.2 | 298 | 332.86 | hom | 332.86 | 333 |
| 1L | 5 | G10 | 187.24 | 187 | 193.5 | 193 | 245.98 | 246 | 248 | 248 | 283.61 | 283 | 290.71 | 290 | 341.4 | 341 | 345.58 | 345 |
| 1N | 5 | H10 | 191.4 | 191 | 193.46 | 193 | 245.96 | 246 | 248 | 248 | 291.11 | 290 | 305.87 | 305 | 337.3 | 337 | 341.5 | 341 |
| | | | | | | | | | | | | | | | | | | |
| 2A | 5 | A11 | 180.95 | 181 | 180.95 | 181 | 248.06 | 248 | 248.06 | 248 | 292.69 | 292 | 302.25 | 302 | 333.02 | 333 | 341.56 | 341 |
| 2L | 5 | B11 | 191.51 | 191 | 193.57 | 193 | 248.03 | 248 | 250.07 | 250 | 283.59 | 283 | 290.77 | 290 | 339.46 | 339 | 339.46 | 339 |
| 2N | 5 | C11 | 187.24 | 187 | 197.69 | 197 | 248.07 | 248 | 248.07 | 248 | 291.23 | 290 | 300.27 | 300 | 337.24 | 337 | 341.49 | 341 |
| LAT2B | 6 | H9 | 187.33 | 187 | 205.9 | 205 | 245.97 | 246 | 248 | 248 | 286.89 | 287 | 290.76 | 290 | 339.38 | 339 | 341.49 | 341 |
| 3A | 5 | D11 | 181.06 | 181 | 181.06 | 181 | 248.01 | 248 | 248.01 | 248 | 292.57 | 292 | 298.17 | 298 | 333 | 333 | 341.47 | 341 |
| 3L | 5 | E11 | 187.18 | 187 | 193.52 | 193 | 245.91 | 246 | 247.96 | 248 | 286.71 | 287 | 290.61 | 290 | 339.37 | 339 | 345.63 | 345 |
| 3N | 5 | F11 | 187.31 | 187 | 191.46 | 191 | 245.95 | 246 | 250.11 | 250 | 291.07 | 290 | 305.85 | 305 | 337.21 | 337 | 341.5 | 341 |
| 5A | 5 | B12 | 181.03 | 181 | 181.03 | 181 | 247.98 | 248 | 247.98 | 248 | 292.59 | 292 | 302.25 | 302 | 333.01 | 333 | 345.7 | 345 |
| 5L | 5 | C12 | 191.47 | 191 | 205.91 | 205 | 247.99 | 248 | 250.13 | 250 | 286.84 | 287 | 292.57 | 292 | 339.36 | 339 | 341.44 | 341 |
| 5N | 5 | D12 | 191.47 | 191 | 197.61 | 197 | 241.79 | 241 | 248.02 | 248 | 283.58 | 283 | 305.8 | 305 | 332.96 | 333 | 337.24 | 337 |

| ALC X LAT | | | Blue | | | | Red | | | | Black | | | | Green | | | | |
|---------------|-------|------|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|-----|-----------|
| Offspring | | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | Allele 1 | | Allele 2 | | |
| Sample | Plate | Well | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Size | int | Paternity |
| 4.4.1.1 | 5 | H12 | 193.58 | 193 | 199.74 | 199 | 248.04 | 248 | 250.13 | 250 | 287.01 | 287 | 292.59 | 292 | 333.12 | 333 | 339.56 | 339 | 4L |
| 4.4.1.2 | 6 | A1 | 193.54 | 193 | 199.74 | 199 | 247.96 | 248 | 247.96 | 248 | 286.87 | 287 | 292.6 | 292 | 333.02 | 333 | 345.72 | 345 | 4L |
| 4.4.1.3 | 6 | B1 | 181.07 | 181 | 191.51 | 191 | 243.93 | 243 | 247.99 | 248 | 283.57 | 283 | 296.38 | 296 | 341.57 | 341 | 345.67 | 345 | 4L |
| 4.4.1.4 | 6 | C1 | 193.43 | 193 | 199.73 | 199 | 248.02 | 248 | 248.02 | 248 | 283.67 | 283 | 296.4 | 296 | 333.05 | 333 | 345.67 | 345 | 4L/4N |
| 4.4.1.5 | 6 | D1 | 191.56 | 191 | 199.82 | 199 | 243.87 | 243 | 250.12 | 250 | 283.59 | 283 | 286.94 | 287 | 341.66 | 341 | 345.75 | 345 | 4L? |
| 4.5.1.2 | 6 | E7 | 180.99 | 181 | 205.8 | 205 | 247.99 | 248 | 250.04 | 250 | 286.92 | 287 | 296.44 | 296 | 339.28 | 339 | 339.28 | 339 | 5L |
| 4.5.1.3 | 6 | F7 | 180.94 | 181 | 205.82 | 205 | 243.83 | 243 | 250.08 | 250 | 292.52 | 292 | 292.52 | 292 | 339.29 | 339 | 343.49 | 343 | 5L |
| 4.5.1.4 | 6 | G7 | 181 | 181 | 191.44 | 191 | 247.94 | 248 | 250.06 | 250 | 286.88 | 287 | 296.36 | 296 | 339.37 | 339 | 341.5 | 341 | 5L |
| 4.5.1.5 | 6 | H7 | 180.82 | 181 | 205.83 | 205 | 243.88 | 243 | 250.04 | 250 | 286.91 | 287 | 292.63 | 292 | 339.39 | 339 | 341.46 | 341 | 5L |
| 4.5.2.1 | 6 | C10 | 180.99 | 181 | 180.99 | 181 | 247.95 | 248 | 247.95 | 248 | 296.39 | 296 | 302.14 | 302 | 341.45 | 341 | 345.6 | 345 | 5A |
| 4.5.2.2 | 6 | A12 | 181.16 | 181 | 199.74 | 199 | 243.88 | 243 | 248.02 | 248 | 286.95 | 287 | 292.74 | 292 | 333.17 | 333 | 345.78 | 345 | 5A |
| 4.5.2.3 | 6 | D9 | 180.86 | 181 | 199.73 | 199 | 243.9 | 243 | 247.99 | 248 | 292.63 | 292 | 296.36 | 296 | 332.86 | 333 | 341.33 | 341 | 5A? |
| 4.5.3.1 | 6 | E11 | 180.91 | 181 | 191.35 | 191 | 248.01 | 248 | 248.01 | 248 | 286.86 | 287 | 296.36 | 296 | 339.19 | 339 | 339.19 | 339 | 5L |
| 4.6.1.1 | 6 | H5 | 181.11 | 181 | 191.54 | 191 | 245.9 | 246 | 245.9 | 246 | 300.09 | 300 | 300.09 | 300 | 343.58 | 343 | 343.58 | 343 | 6N |
| 4.6.1.2 | 6 | A6 | 181 | 181 | 191.43 | 191 | 245.93 | 246 | 247.95 | 248 | 291.03 | 290 | 296.31 | 296 | 332.94 | 333 | 341.49 | 341 | 6N |
| 4.6.1.3 | 6 | B6 | 180.97 | 181 | 180.97 | 181 | 243.84 | 243 | 245.9 | 246 | 292.48 | 292 | 296.33 | 296 | 339.27 | 339 | 343.49 | 343 | 6A/6L |
| 4.6.1.4 | 6 | C6 | 180.9 | 181 | 193.51 | 193 | 245.97 | 246 | 248.04 | 248 | 286.77 | 287 | 300.09 | 300 | 332.85 | 333 | 339.27 | 339 | 6L |
| 4.6.1.5 | 6 | D6 | 180.9 | 181 | 191.4 | 191 | 245.88 | 246 | 247.95 | 248 | 300 | 300 | 300 | 300 | 337.15 | 337 | 343.53 | 343 | 6N |
| Female | | | | | | | | | | | | | | | | | | | |
| 4.5.1 | 5 | B10 | 180.86 | 181 | 180.86 | 181 | 243.9 | 243 | 248.08 | 248 | 292.63 | 292 | 296.45 | 296 | 339.28 | 339 | 343.46 | 343 | |
| 4.5.2 | 5 | C10 | 180.96 | 181 | 199.73 | 199 | 243.89 | 243 | 248.02 | 248 | 286.87 | 287 | 296.4 | 296 | 332.84 | 333 | 341.45 | 341 | |
| 4.5.3 | 5 | D10 | 180.76 | 181 | 180.76 | 181 | 243.87 | 243 | 248.03 | 248 | 292.5 | 292 | 296.39 | 296 | 339.27 | 339 | 343.44 | 343 | |
| 4.6.1 | 5 | E10 | 180.92 | 181 | 180.92 | 181 | 245.97 | 246 | 245.97 | 246 | 296.5 | 296 | 300.18 | 300 | 332.89 | 333 | 343.49 | 343 | |
| Male | | | | | | | | | | | | | | | | | | | |
| 4A | 5 | G11 | 180.95 | 181 | 180.95 | 181 | 248.06 | 248 | 248.06 | 248 | 292.53 | 292 | 298.22 | 298 | 341.51 | 341 | 345.68 | 345 | |

| | | | | | | | | | | | | | | | | | | |
|----|---|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|--------|-----|
| 4L | 5 | H11 | 191.5 | 191 | 193.54 | 193 | 248.03 | 248 | 250.04 | 250 | 283.55 | 283 | 292.56 | 292 | 339.47 | 339 | 345.71 | 345 |
| 4N | 5 | A12 | 191.62 | 191 | 197.74 | 197 | 248.04 | 248 | 250.11 | 250 | 296.54 | 296 | 300.27 | 300 | 333.16 | 333 | 337.46 | 337 |
| 5A | 5 | B12 | 181.03 | 181 | 181.03 | 181 | 247.98 | 248 | 247.98 | 248 | 292.59 | 292 | 302.25 | 302 | 333.01 | 333 | 345.7 | 345 |
| 5L | 5 | C12 | 191.47 | 191 | 205.91 | 205 | 247.99 | 248 | 250.13 | 250 | 286.84 | 287 | 292.57 | 292 | 339.36 | 339 | 341.44 | 341 |
| 5N | 5 | D12 | 191.47 | 191 | 197.61 | 197 | 241.79 | 241 | 248.02 | 248 | 283.58 | 283 | 305.8 | 305 | 332.96 | 333 | 337.24 | 337 |
| 6A | 5 | E12 | 181.02 | 181 | 181.02 | 181 | 248.05 | 248 | 248.05 | 248 | 292.57 | 292 | 298.23 | 298 | 332.98 | 333 | 341.5 | 341 |
| 6L | 5 | F12 | 191.46 | 191 | 193.53 | 193 | 248.03 | 248 | 250.06 | 250 | 286.81 | 287 | 290.71 | 290 | 339.37 | 339 | 339.37 | 339 |
| 6N | 5 | G12 | 191.45 | 191 | 193.5 | 193 | 245.94 | 246 | 248.06 | 248 | 291.18 | 290 | 300.27 | 300 | 337.32 | 337 | 341.57 | 341 |

APPENDIX 2:

Trio 1(b?):

ALC:



LAT:



NDL:



Trio 2 (A):

ALC



LAT:



NDL:



Trio 3:

ALC:



LAT:



NDL:



Trio 4:

ALC + NDL



LAT:



Trio 5:

ALC:



LAT:



NDL:



Trio 6:



LAT:



NDL:



APPENDIX 3:

Table 4- Size of all males within each Trio (SL, mm).

| Trio | ALC | LAT | NDL |
|------|-----|-----|-----|
| 1 | 68 | 56 | 61 |
| 2 | 68 | 55 | 64 |
| 3 | 72 | 64 | 64 |
| 4 | 68 | 68 | 66 |
| 5 | 79 | 68 | 65 |
| 6 | 70 | 59 | 65 |

APPENDIX 4:

Table 3. Exposure of Trio to female species tank. *A. alcalica* females were introduced to male Trios 1, 4 and 6. *A. latilabris* with Trios 2 and 3. *A. ndalalani* with Trios 1, 2, 3, and 5. The hybrid *A. alcalica* x *A. latilabris* females were introduced to Trios 4, 5, and 6. This Table also indicates the number of offspring fathered by each male within their Trio. This table shows that within each Trio, conspecifics produced the most offspring. Trio 1 ALC in Tank 3 fathered a close number of offspring to NDL. Within the hybrid tank (Tank 4), LAT males fathered the most offspring, with the exceptions of Trio 6. NDL sired no offspring within Tank 1 or 2. Offspring which were not of certain paternity, due to similarity in paternal allele size was not included but can be observed in Appendix 1.

Number of offspring fathered

| Tank | Female type | Trio1 | | | Trio2 | | | Trio3 | | | Trio4 | | | Trio5 | | | Trio6 | | |
|------|--------------|-------|-----|-----|-------|-----|-----|-------|-----|-----|-------|-----|-----|-------|-----|-----|-------|-----|-----|
| | | ALC | LAT | NDL | ALC | LAT | NDL | ALC | LAT | NDL | ALC | LAT | NDL | ALC | LAT | NDL | ALC | LAT | NDL |
| 1 | ALC | 8 | 1 | 0 | - | - | - | - | - | - | 5 | 1 | 0 | - | - | - | 2 | 0 | 0 |
| 2 | LAT | - | - | - | 1 | 2 | 0 | 1 | 4 | 0 | - | - | - | - | - | - | - | - | - |
| 3 | NDL | 3 | 0 | 4 | 0 | 1 | 2 | 0 | 1 | 3 | - | - | - | 0 | 0 | 24 | - | - | - |
| 4 | ALC x LAT | - | - | - | - | - | - | - | - | - | 0 | 4 | 0 | 1 | 5 | 0 | 0 | 1 | 3 |

