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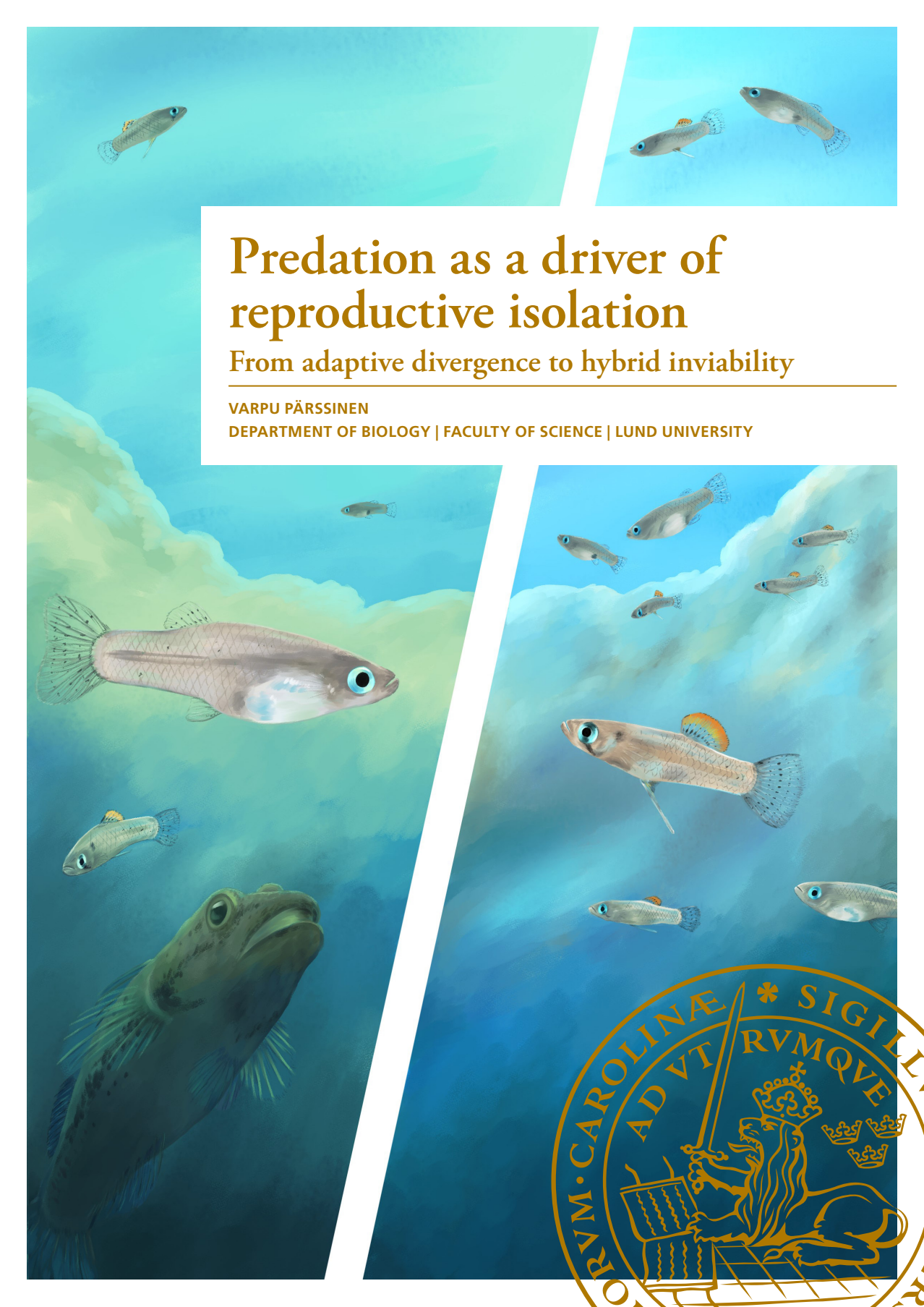
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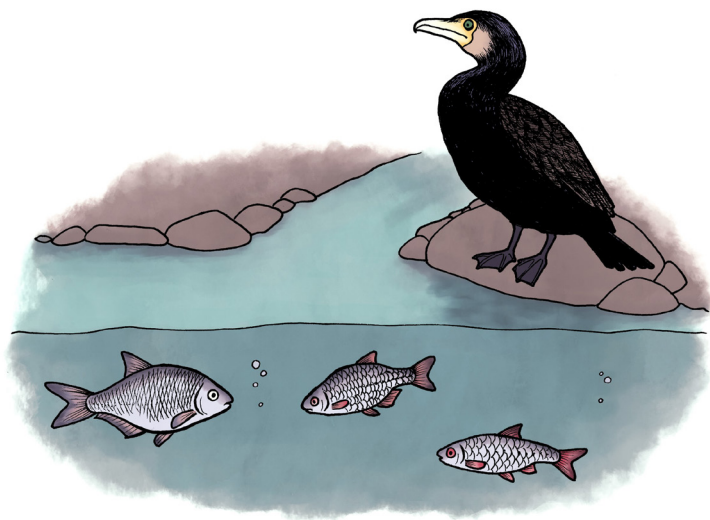
Predation as a driver of reproductive isolation

From adaptive divergence to hybrid inviability

VARPU PÄRSSINEN

DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY





List of papers

- I. Pärssinen, V., Hulthén, K., Brönmark, C., Björnerås, C., Ekelund Ugge, G., Gollnisch, R., Hansson, L.-A., Herzog, S.D., Hu, N., Johansson, E., Lee, M., Rengefors, K., Sha, Y., Škerlep, M., Vinterstare, J., Zhang, H., Langerhans, R.B. and Nilsson, P.A. 2021. Variation in predation regime drives sex-specific differences in mosquitofish foraging behaviour. *Oikos* 130:790-797.
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- IV. Pärssinen, V., Hulthén, K., Brönmark, C., Skov, C., Brodersen, J., Baktoft, H., Chapman, B.B., Hansson, L.-A., and Nilsson, P.A. 2020. Maladaptive migration behaviour in hybrids links to predator-mediated ecological selection. *Journal of Animal Ecology* 89:2596-2604.

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Varpu Pärssinen



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DOCTORAL DISSERTATION

by due permission of the Faculty of Science, Lund University, Sweden.
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Professor Anna Qvarnström
Uppsala University

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Abstract <p>Natural selection can play an important role in the origin of new species. When reproductive isolation evolves as a result of ecologically-based divergent natural selection, the process is referred to as ecological speciation. In most organisms, sufficient reproductive isolation is considered to be essential for the establishment of new species. However, reproductive isolation typically involves multiple isolating barriers, and we still lack knowledge of how some barriers are affected by the level of ecological divergence and the stage of speciation.</p> <p>The aim of this thesis is to estimate how a potent source of ecological selection, predation, may drive reproductive isolation. First, I study multiple isolated populations of Bahamas mosquitofish (<i>Gambusia hubbsi</i>), that have adapted to either of two distinct selective regimes depending on the presence or absence of predatory fish. I estimate how the behaviour of mosquitofish has diverged under these contrasting predation regimes, and whether reproductive isolation has evolved to be stronger between populations from different predation regimes. Second, I study the behavioural phenotype of naturally occurring hybrids between roach (<i>Rutilus rutilus</i>) and common bream (<i>Abramis brama</i>), and ask if maladaptive behaviour can underlie increased susceptibility to predation in hybrids.</p> <p>Through behavioural trials using either wild-caught or laboratory-reared Bahamas mosquitofish, I show that key behavioural traits have diverged between predation regimes, but also highlight that the degree of divergence is often sex specific. I found that low-predation environments may select for foraging traits that are likely beneficial under high resource competition, but this effect was only seen in the female sex. I also show that male, but not female, mating behaviours have diverged between predation regimes, as males adapted to a high-predation regime courted females and attempted to initiate mating more actively in comparison to males originating from low-predation environments. By comparing behavioural traits expressed during within-population and between-population mating trials, I discovered that females were more aggressive towards foreign males and initiated aggression faster towards males from the opposite predation regime. I thus show that ecological adaptation may act to strengthen behavioural isolation between populations in this system.</p> <p>Furthermore, I show that hybrids produced between parents originating from different predation regimes have the lowest survival rates out of all hybrids and pure-line offspring reared in common-garden conditions. This indicates that ecological divergence can lead to intrinsic hybrid incompatibilities relatively early along the speciation continuum. The hybrid crosses between different predation regimes also showed intermediate fast-start escape-performance abilities when compared to crosses within the same predation regime, indicating that hybrids produced between divergently adapted populations may have lower ecological viability in the high-predation environment. Monitoring of the migration patterns through passive telemetry revealed that roach × bream hybrids show a higher frequency of migratory trips between the stream and lake habitats, a behaviour that exposes them to a higher predation risk. I thus show that predation can reinforce species integrity by selecting against hybrid phenotypes.</p> <p>In summary, differences in predation risk between populations can lead to stronger reproductive isolation as a by-product of adaptive divergence. Furthermore, several forms of pre- and postzygotic isolation may be important for this process, even at an early stage of speciation. Finally, predation may also play an important role in the later stages of speciation as a reinforcer of species integrity.</p>			
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Author contributions

I. VP, KH, ChB, RBL and PAN conceived and led the study. VP performed the field experiment with the help of KH, ChB, CaB, GEU, RG, LAH, SDH, NH, EJ, ML, KR, YS, MŠ, JV, HZ, RBL and PAN. VP collected the data from video recordings, analysed the data and drafted the first version of manuscript. Study was done as a part of a PhD student trip, where all authors helped to plan the study, read the manuscript and provided guidance and revisions.

II. VP, KH, RBL and PAN conceived and designed the study. VP and KH performed the laboratory trials with the help of RBL. VP collected the data from video recordings, analysed the data and wrote the first draft of the manuscript. PAN and KH provided comments to the manuscript. All authors contributed significantly to the final version of the manuscript.

III. VP, KH, RBL and PAN conceived and designed the study. VP and RBL performed the laboratory trials on hybrids, while parental fast-start trials were performed by KH and RBL. VP collected the data from video recordings and analysed the data concerning fast-start and foraging. RBL led the data analyses concerning hybrid survival with contributions from VP. VP wrote the first draft of the manuscript. All authors read the manuscript and provided guidance and revisions.

IV. CS and HB collected the field data. VP analysed the data and led the writing of the manuscript with the help of KH, ChB and PAN. CS, JB, HB, BBC and LAH contributed substantially to the design and revisions of the study.

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Abstract

Natural selection can play an important role in the origin of new species. When reproductive isolation evolves as a result of ecologically-based divergent natural selection, the process is referred to as ecological speciation. In most organisms, sufficient reproductive isolation is considered to be essential for the establishment of new species. However, reproductive isolation typically involves multiple isolating barriers, and we still lack knowledge of how some barriers are affected by the level of ecological divergence and the stage of speciation.

The aim of this thesis is to estimate how a potent source of ecological selection, predation, may drive reproductive isolation. First, I study multiple isolated populations of Bahamas mosquitofish (*Gambusia hubbsi*), that have adapted to either of two distinct selective regimes depending on the presence or absence of predatory fish. I estimate how the behaviour of mosquitofish has diverged under these contrasting predation regimes, and whether reproductive isolation has evolved to be stronger between populations from different predation regimes. Second, I study the behavioural phenotype of naturally occurring hybrids between roach (*Rutilus rutilus*) and common bream (*Abramis brama*), and ask if maladaptive behaviour can underlie increased susceptibility to predation in hybrids.

Through behavioural trials using either wild-caught or laboratory-reared Bahamas mosquitofish, I show that key behavioural traits have diverged between predation regimes, but also highlight that the degree of divergence is often sex specific. I found that low-predation environments may select for foraging traits that are likely beneficial under high resource competition, but this effect was only seen in the female sex. I also show that male, but not female, mating behaviours have diverged between predation regimes, as males adapted to a high-predation regime courted females and attempted to initiate mating more actively in comparison to males originating from low-predation environments. By comparing behavioural traits expressed during within-population and between-population mating trials, I discovered that females were more aggressive towards foreign males and initiated aggression faster towards males from the opposite predation regime. I thus show that ecological adaptation may act to strengthen behavioural isolation between populations in this system.

Furthermore, I show that hybrids produced between parents originating from different predation regimes have the lowest survival rates out of all hybrids and pure-line offspring reared in common-garden conditions. This indicates that ecological divergence can lead to intrinsic hybrid incompatibilities relatively early along the speciation continuum. The hybrid crosses between different predation regimes also showed intermediate fast-start escape-performance abilities when compared to crosses within the same predation regime, indicating that hybrids produced between

divergently adapted populations may have lower ecological viability in the high-predation environment. Monitoring of the roach \times bream hybrid migration patterns through passive telemetry revealed that hybrids show a higher frequency of migratory trips between the stream and lake habitats, a behaviour that exposes them to a higher predation risk. I thus show that predation can reinforce species integrity by selecting against hybrid phenotypes.

In summary, differences in predation risk between populations can lead to stronger reproductive isolation as a by-product of adaptive divergence. Furthermore, several forms of pre- and postzygotic isolation may be important for this process, even at an early stage of speciation. Finally, predation may also play an important role in the later stages of speciation as a reinforcer of species integrity.

Popular science summary

There are millions of unique species in the world, but we are still discovering new ways of how species can be born. Over the course of evolution, a single species can start to develop in two different directions, until eventually, these two lineages may turn into two separate species. Generally, species are considered distinct from each other when they fail to reproduce with one another, or if their hybrid offspring are unable to mature and reproduce successfully. Many types of barriers can evolve to prevent successful breeding between closely related lineages, but together, they are referred to as reproductive isolation.

Biologists have known for some time that reproductive isolation can evolve as a result of a group of organisms adapting to two different environments. This process is called ecological speciation. For example, if an insect species specialises in eating only two different species of plants, successful mating may happen more commonly with insect individuals that eat the same type of plant. However, we still do not know how much some types of natural selection, like predation, can lead to different forms of reproductive isolation. Variation in animals that are in the middle of the speciation process is an important part of the diversity of life, so more knowledge on how this process can proceed or reverse is essential in today's changing world.

In my thesis, I studied how prey may adapt to different levels of predation pressure, and how this may affect different forms of reproductive isolation. A large part of my thesis focuses on the Bahamas mosquitofish, a small fish that gives birth to live young. On the Bahamas islands, you can find many inland blue holes, which are collapsed caves filled with water. Many of these blue holes give home to Bahamas mosquitofish communities, which have stayed fairly isolated from each other since the last ice age. However, some blue holes also have a larger fish species, the bigmouth sleeper, that preys on the mosquitofish. The mosquitofish have therefore evolved in two distinct predation regimes: one with a constant high risk of being eaten by a predator, and the other where the lack of major predators has led to high mosquitofish densities, and as a consequence, higher competition for resources such as food.

Few studies have looked into what kind of prey behaviour might be beneficial in an environment without major predators. By recording the feeding behaviour of wild-caught mosquitofish in the laboratory, I found that females from the low-predation environment search and eat more food than females from the high-predation environment in the same amount of time. Males, on the other hand, showed weaker differences. Female mosquitofish might respond to the highly competitive pressure of the low-predation regime more easily, since they need to grow larger than the males.

Using mosquitofish that had been grown in the laboratory, I studied how easily mosquitofish from different blue holes were able to reproduce with each other,

expecting that reproduction might be especially hard between mosquitofish that descended from different predation regimes. I first studied if the mosquitofish showed differences in their mating behaviour depending on which predation regime they descended from, or what kind of mate they were paired with. The behaviour of male mosquitofish depended consistently on their predation regime of origin, but not on the female origin. Females, on the other hand, did not differ in their behaviour between predation regimes, but they were generally more aggressive towards males from different blue holes than their own, especially if that blue hole had a different predation regime from theirs. In the wild, females might therefore resist male mating attempts especially strongly if the male had arrived from another blue hole with different level of predation risk. I also monitored if the females became pregnant after they had interacted with a male, and found that matchups of mosquitofish between different predation regimes had a lower chance of resulting in offspring. This further confirms that the ecological differences between these two predation regimes have created barriers that prevent the fish from mating successfully.

I raised the hybrid offspring between different blue holes into adulthood in the laboratory. Here, I discovered that compared to offspring born within the native blue hole community, the hybrids parented by individuals from different predation regimes died more often before reaching maturity. It is likely that genes of the mosquitofish have changed into different directions between the two predation regimes, and these results show that the genetic differences can result in innate problems in the offspring born between the different regimes. I also tested how well the remaining hybrid offspring might perform in either high-predation or low-predation environment. On average, the hybrid offspring produced between different predation regimes showed a lower ability to escape predators than fish produced within the high-predation, but did show higher escape abilities than low-predation fish. In the wild, hybrids born between a native high-predation fish and a low-predation immigrant may thus get caught by predators more easily than native fish. On the other hand, I did not detect strong differences in foraging behaviour between hybrids, regardless of their origin.

For the final part of my thesis, I studied the migration behaviour of roach, bream and their hybrids in a Danish lake. In this area, some roach and bream migrate from the lakes into the connecting rivers for the winter. While the hybrids are intermediate between roach and bream in many ways, I found that they also travel between the river and lake many more times within a single season than either roach or bream. This novel behaviour also seemed to make them more likely to be eaten by cormorants. Therefore, predation by cormorants may help to reduce the number of hybrids, thus assisting that roach and bream stay as separate species.

In summary, my thesis shows that differences in predation pressure can often drive the evolution of prey. Since predation contributed to many different forms of reproductive isolation, these barriers can act together and lead to birth of new species.

Suomenkielinen tiivistelmä

Maailmasta on löydetty miljoonia lajeja, mutta emme edelleenkään tiedä kaikkea niistä askeleista, jotka johtavat uusien lajien syntymään. On kuitenkin selvää, että jos yksi laji alkaa jostain syystä jakautua kahteen eri linjaan, tuloksena saattaa olla lopulta kaksi erillistä lajia. Laji määritellään yleensä niin, että eri lajien edustajat eivät kykene lisääntymään keskenään, tai niiden välisestä risteymästä syntyneet jälkeläiset eivät kykene lisääntymään normaaliin tapaan. Eriytyvien eliöryhmien välille voi kehittyä monenlaisia lisääntymisestiteitä, joita kutsutaan myös isolaatiomekanismeiksi. Yhdessä nämä lisääntymisestit voivat eristää lähisukuiset eliöryhmät toisistaan niin tehokkaasti, että niitä aletaan pitää erillisinä lajeina.

Biologit ovat jo jonkin aikaa tienneet, että jos saman lajin edustajia sopeutuu kahteen erilaiseen elinympäristöön, näiden ryhmien välille saattaa kehittyä enemmän lisääntymisestiteitä kuin samanlaiseen ympäristöön sopeutuneiden ryhmien välille. Tätä kutsutaan ekologiseksi lajiutumiseksi. Jos hyönteislaji esimerkiksi erikoistuu syömään vain kahta eri kasvilajia, parittelu saattaa onnistua helpommin kahden samanlaista kasvia syövän yksilön välillä. Emme silti tiedä kovin paljoa siitä, kuinka jotkin luonnonvalinnan muodot, kuten saalistus, vaikuttavat eri lisääntymisestiteiden kehittymiseen. Lajien sisällä tapahtuva vaihtelu ja vielä keskeneräinen lajiutuminen ovat tärkeä osa nykyistä elonkirjoa, joten on tärkeää tutkia, missä olosuhteissa ne syntyvät ja milloin ne saattavat hävitä. Tämä on erityisen oleellista nyt, kun huippupetojen määrä on ihmisen vaikutuksesta kokenut suuria muutoksia ympäri maailmaa.

Väitöskirjassani tutkin kuinka saalislajit sopeutuvat eritasoisiin saalistuspaineisiin, ja kuinka tämä saattaa johtaa eri tyyppisiin isolaatiomekanismeihin. Suurin osa työstäni keskittyy pieneen eläviä poikasiesynnyttävään kalalajiin, bahamalaiseen moskiittokalaan. Karibialaisilla Bahamaan kuuluvilla saarilla esiintyy suuri määrä niin sanottuja ”sinisiä aukkoja”, meriveden täyttämiä romahtaneita luolia. Sisämaan siniset aukot ovat kuin toisistaan eristyneitä syviä järviä, ja monessa niistä on elänyt oma moskiittokalapopulaationsa viime jääkaudelta asti. Joissakin sinisissä aukkoissa elää kuitenkin myös petokaloja. Näin ollen moskiittokalat ovat saaneet kehittyä kahdessa vaihtoehtoisessa ympäristössä: korkean saalistuspaineen populaatioissa kalat ovat jatkuvasti vaarassa joutua pedon suihin, kun taas matalan petopaineen populaatioissa saalistusta ei juuri tapahdu, ja siitä seuraavan korkean populaatiotiheyden vuoksi moskiittokalat joutuvat kilpailemaan ruuasta keskenään.

Saaliseläinten käyttäytymistä petojen läsnä ollessa on tutkittu paljon, mutta petojen puuttumisen synnyttämä valintapaine on jäänyt vähemmälle huomiolle. Luonnonkalojen havainnointi laboratoriossa paljasti, että moskiittokalanaaraat etsivät ja syövät ruokaa tiheämmällä tahdilla, jos ne ovat sopeutuneet matalaan saalistuspaineeseen. Koirailta yhtä vahvaa eroa ei löytynyt. Matalan saalistuspaineen

aiheuttama intensiivinen kilpailu saattaakin vaikuttaa naaraiden käyttäytymiseen voimakkaammin, sillä niiden täytyy kasvaa koiraita suuremmiksi.

Tutkimuksissani selvitin kuinka helposti eri populaatioista peräisin olevat moskiittokalat pystyvät risteytymään laboratoriossa. Jos ekologista lajiutumista olisi ehtinyt tapahtua, oletin lisääntymismenestyksen olevan erityisen alhaista eri saalistuspaineisiin sopeutuneiden populaatioiden välillä. Ensin keskityin kalojen pariutumiskäyttäytymiseen, tutkien kuinka naaraiden ja koiraiden käytös riippuu sekä niiden alkuperäisestä elinympäristöstä, että niille annetun parin alkuperästä. Selvisi, että koiraiden käyttäytyminen riippuu niiden alkuperäisestä petoympäristöstä, mutta naaraan alkuperällä ei ollut niille selväsuuntaista vaikutusta. Naaraiden käytöstä sen sijaan sääтели ainoastaan koiraan alkuperä. Naaraat olivat nimittäin aggressiivisempia oman populaationsa ulkopuolisia koiraita kohtaan, erityisesti jos kyseinen koiras oli erilaisesta petoympäristöstä kuin naaras. Luonnossa naaraat saattavatkin vastustaa koiraan paritteluyrityksiä voimakkaammin, jos koiras on saapunut eri saalistuspaineen elinympäristöstä. Seurasin myös kuinka suuri osa naaraista tuli kantavaksi koiraan kohdattuaan. Tästä selvisi, että kalapareilla, jotka olivat lähtöisin eri saalistuspaineiden ympäristöistä, oli todellakin huonoin todennäköisyys saada onnistuneesti poikasia keskenään.

Kasvatin eri populaatioiden välisiä risteymiä lisääntymisikäisiksi laboratorioolosuhteissa. Tällöin selvisi, että eri petopaineen alla olevien populaatioiden välisistä risteymistä syntyvät jälkeläiset kuolivat jo kasvuvaiheessa selvästi useammin kuin saman populaation sisällä syntyneet poikaset. Moskiittokalojen perimä on todennäköisesti kehittynyt eri suuntiin niiden elinympäristön saalistuspaineesta riippuen. Poikasten korkea kuolleisuus osoittaakin, että niiden vanhempien perimät eivät ole enää kaikin osin yhteensopivia, jolloin risteymissä saattaa esiintyä vakavia kehityshäiriöitä. Lisäksi tutkin, kuinka aikuisuuteen kasvaneet kalaristeyvät saattaisivat pärjätä luonnossa joko korkean tai matalan saalistuspaineen ympäristössä. Eri petoympäristöjen välillä syntyneet risteyvät suoriutuivatkin melko keskiverroksi: ne osoittivat kykyä paeta saalistajia paremmin kuin matalan saalistuspaineen sisäiset risteyvät, mutta eivät olleet yhtä hyviä pakoreaktiossa kuin korkean saalistuspaineen populaatioiden välillä syntyneet yksilöt. Luonnossa eri saalistuspaine ympäristöjen välillä syntyneet poikaset voivatkin jäädä helpommin petojen suihin. Ruoan etsimis- tai syömistahdissa taas ei näyttänyt olevan suuria eroja eri risteymätyyppien välillä.

Tämän kirjan viimeisessä luvussa tutkin särjen ja lahnan risteymien, ns. ”särkilahnojen” käyttäytymistä tanskalaisessa järvestä. Osa alueen särki- ja lahnakannasta muuttaa talvella järvestä ympäröiviin jokiin. Vaikka särkilahnat ovat yleensä ulkomuodoltaan täysin särjen ja lahnan välimaastosta, tutkimuksessa havaittiin, että ne tekevät muuttomatkoja edestakaisin järven ja joen välillä huomattavasti enemmän saman syksyn aikana kuin särjet tai lahnat. Tämä poikkeava käyttäytyminen näytti myös aiheuttavan sen, että särkilahnat jäivät helpommin merimetson saaliiksi. Merimetsojen

aiheuttama saalistuspaine saattaa siis auttaa estämään särjen ja lahnan risteytymistä, pitäen näin lajit erillään toisistaan.

Väitöskirjani tulokset osoittavat, että saalistuspaineen vaihtelevuus eri alueiden välillä voi johtaa saalislajeissa monen eri tyypin isolaatiomekanismeihin. Lisääntymisesteiden voimakkuus näyttää silti vaihtelevan alkuperäisestä saalistusympäristöstä ja yksilöiden sukupuolesta riippuen. Yhdessä nämä lisääntymisesteet voivat kuitenkin johtaa jopa alkuperäisen saalislajin jakautumiseen useaksi uudeksi lajiksi.

Introduction

Over a million species have been described in the world, but the mechanisms by which species are shaped and maintained are still intensively discussed. Even the concept of a species has been defined in a multitude of different ways, as almost any attempt at a strict definition is met with an exception that breaks the rule (Coyne and Orr 2004). Still, in sexually reproducing organisms, it is generally agreed that distinct species need to have some form of reproductive isolation, which substantially limits them from interbreeding with other groups of organisms. Once even a weak reproductive barrier has appeared, the speciation process continues as long as these barriers (and, thus, overall reproductive isolation) build up over time. In the words of Coughlan and Matute (2020), “*reproductive barriers are the currency of speciation*”, and the accumulation of this currency defines how far populations are on the speciation continuum (Stankowski and Ravinet 2021). Thus, in order to understand how speciation occurs, it is essential to study how reproductive isolation first appears and how fast it accumulates. This is not a simple task, especially since speciation rates greatly vary between taxa (Near *et al.* 2013, Rabosky 2016, Scholl and Wiens 2016). The rate at which reproductive isolation builds up can be influenced by intrinsic factors such as mutation rate (Lanfear *et al.* 2010), but given that we have not been able to identify any single factor responsible for the vast diversity on earth, there are likely multiple forces acting simultaneously. Could extrinsic factors then, such as interactions between organisms or their environment, also facilitate the build-up of reproductive barriers?

Ever since Darwin’s *On the Origin of Species* (1859), biologists have generally agreed that natural selection plays an important role in the emergence of new species. Despite this, the mechanisms by which selection could start and accelerate the speciation process received fairly little attention in the early decades of speciation research. More attention instead went into the importance of biogeographical context. Geography often plays a role in speciation, as the speciation process is most commonly expected to start after populations go through a period of allopatry, i.e. where the gene flow between populations is cut off by a geographical barrier (Coyne and Orr 2004). This allows small incompatibilities to emerge, either through selection or genetic drift, and once enough differences between populations have accumulated, the reproductive isolation may keep the populations from interbreeding even if the geographical barrier disappears. However, over time more studies started to suggest that speciation may be possible with some gene flow or even in sympatry (i.e. within one population with no

geographical barriers), as long as strong selection for divergent ecological adaptation drives individuals to mate with only part of the population (Rice and Salt 1990, Johnson *et al.* 1996). One of the most classic examples of this is the apple maggot (*Rhagoletis pomonella*), which has diverged into using either hawthorn or domestic apple as its host plant, and these two host adaptations rarely mix because the flies mate next to the fruit of their host plant (Filchak *et al.* 2000). Ecology may thus play a strong role in speciation in a variety of different biogeographical contexts. Still, only during the last two decades has the interest in and evidence for the role of divergent selection in speciation truly grown.

Ecological speciation and its alternatives

Modern studies have highlighted the role of ecology in the speciation process, showing that divergent ecological adaptation can lead to the evolution of reproductive isolation (Rundle and Nosil 2005, Nosil 2012). This is generally referred to as ecological speciation. The ecological forces that may cause divergent selection come in many forms, such as available habitat or host types, resource availability, strength of competition, and intensity of parasitism or predation. For example, *Timema* stick insects have several colour morphs that differ in host plant use, and reproductive isolation seems to be stronger between different colour morphs than in similar colour morphs using the same host plant (Nosil *et al.* 2003). Similar patterns may hold even across multiple species pairs, like the stickleback species studied by Lackey and Boughman (2017). They found ecological divergence to be the best predictor of total level of reproductive isolation between species pairs, instead of time since species divergence or genetic distance. On an even wider scale, a comparative study across > 500 species pairs in plants, invertebrates and vertebrates showed that ecological divergence was positively associated with the level of reproductive isolation regardless of taxa, ecological trait or type of reproductive barrier (Funk *et al.* 2006). Thus, there seems to be no doubt that divergent ecological adaptation is important for speciation on a large scale. However, we still lack a lot of knowledge of the specific mechanisms with which natural selection might facilitate the build-up of reproductive isolation.

Ecological speciation can happen in any geographical context (Rundle and Nosil 2005), but geography limits by which mechanism ecology can limit gene flow. The possible mechanisms are connected to the two common explanations as to why speciation occurs in the first place (Maynard Smith and Szathmáry 1995, Coyne and Orr 2004). The first explanation assumes that if populations evolve in different directions through natural selection, they will eventually become incompatible as a by-product of these differences. For example, Dodd (1989) demonstrated that after rearing isolated *Drosophila* populations on two different food sources for several generations in the lab,

the flies developed a preference to mate with individuals that had been reared on the same food source as themselves. The second explanation assumes that diverging populations maximize their respective reproductive outputs (fitness) by occupying different ecological niches. These niches can be seen as adaptive fitness peaks in an adaptive landscape, where individuals in valleys are unfit for the current environment (Gavrilets 2004). Thus, if individuals on different fitness peaks were to interbreed, their offspring would likely fall between fitness peaks (Figure 1). Selection can therefore favour individuals that breed only with individuals from the same fitness peak, and, consequently, reproductive isolation forms between the peaks. In sympatry (or parapatry, where gene flow is present but partially limited), both mechanisms can possibly take place, and distinguishing between them is often challenging. On the other hand, it has even been argued that speciation is only completed after selection has the chance to select against the production of unfit hybrids, which seems to lead to enhanced reproductive isolation in sympatry (Blair 1974, Schluter and McPhail 1992, Rundle and Nosil 2005). The prevalence of this process, referred to as reinforcement, still remains under debate (Hollander *et al.* 2018). In allopatric populations, however, reproductive isolation can only form through the by-product mechanism. This is because in allopatry, virtually no offspring are being formed between the populations, and therefore natural selection cannot act against individuals that produce unfit hybrids. Thus, if reproductive isolation is found between allopatric populations that have not been in contact since the original divergence, there are fewer possible mechanisms that could have caused the isolation to emerge.

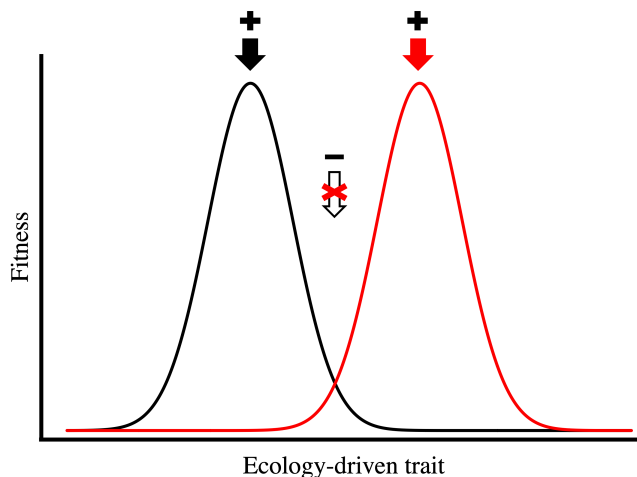


Figure 1. Divergent ecological selection results in two different fitness peaks, driving populations apart. On average, hybrids produced between these peaks will fall into a fitness valley, and thus, hybridization is selected against.

The alternative of ecological speciation is often called mutation-order speciation. In this process, populations adapt to a similar selection pressure, but reproductive isolation evolves as a result of different adaptive alleles becoming fixated by chance (Mani and Clarke 1990, Schluter 2009). These terms have received some critique since ecology technically plays a role in both types of speciation (Sobel *et al.* 2010), and it has been suggested that they may be more intuitively referred to as ‘speciation by divergent selection’ (as opposed to ecological speciation) and ‘speciation by similar selection’ (as opposed to mutation-order) instead (Langerhans and Riesch 2013). However, I will stick to the most well-known terms here for the sake of clarity. Since the mutation-order mechanism occurs as a response to selection, it has potential to lead to reproductive isolation at a faster rate than simple genetic drift. While few concrete examples of mutation-order speciation have been found so far, there are many examples of organisms achieving the same phenotype through different genotypes, so its potential effect on speciation cannot be ignored. For example, multiple populations of beach mice have evolved divergent colour morphs as camouflage to darker mainland soil and lighter coastal sand, but populations in the same environment have achieved the similar colours through mutations in different loci (Steiner *et al.* 2009). If these changes in different loci created genetic incompatibilities that resulted in reproductive isolation, it would be a case of speciation through adaptations to uniform selection, i.e. mutation-order speciation. As studies comparing speciation between environments under divergent selection and uniform selection are scarce, it remains unclear how prevalent mutation-order speciation is in comparison to ecological speciation.

Effect of ecology on different reproductive barriers

Reproductive isolation can be divided into many types of reproductive barriers, which may act alone or simultaneously depending on the system. The isolating barriers are typically grouped into two categories: barriers that take place before the gametes from the female and the male form a zygote (prezygotic) and barriers that take place after a hybrid zygote is formed (postzygotic). The distinction is useful for discerning which individuals are under selection, as prezygotic isolation depends on the traits of the parents, and postzygotic isolation depends on the traits of the hybrid offspring. Reproductive barriers can also be categorized into pre- and postmating isolation, where premating barriers take place before successful gamete transfer (most prezygotic barriers) and postmating barriers after mating (gametic isolation, all postzygotic barriers). Here, I briefly discuss reproductive barriers in the context of ecological divergence, and if studied, by which mechanism they are expected to emerge.

Prezygotic isolation

Ecological isolation can limit gene flow by spatially or temporally preventing individuals from mating (Dambroski and Feder 2007, Bolnick *et al.* 2009, Chin and Cristescu 2021). In *habitat isolation*, individuals have genetically determined preferences for mating in different habitats, whereas in temporal isolation, individuals mate at different times. These preferences typically develop through divergent ecological adaptation (reviewed in Rundle and Nosil (2005)), such as preference for different host plants (Funk *et al.* 2002, Egan and Funk 2006). Depending on the system, both sympatry (Rybinski *et al.* 2016) and allopatry (Nosil *et al.* 2006) can lead to stronger divergence in habitat use, so habitat isolation may develop through both a reinforcement-like process and as a by-product of selection. However, as few other examples exist so far, final conclusions of the mechanism behind ecological isolation are hard to draw.

Gene flow between ecologically divergent populations can also be reduced by migrants having reduced viability in their non-native habitat. This “*immigrant inviability*” has only recently been considered its own form of reproductive isolation, but there is already evidence that it can act as a major barrier between populations (Nosil *et al.* 2005, Lowry *et al.* 2008). For example, some populations of the fish *Poecilia mexicana* have adapted to sulfidic waters, and non-adapted immigrants to such habitats survive poorly (Plath *et al.* 2013). Immigrant inviability is considered to take place only between ecologically diverged populations, and thus it may be a strong contributor to ecological speciation (Nosil *et al.* 2005).

Behavioural isolation (a.k.a. sexual isolation) is a well-studied prezygotic reproductive barrier, where individuals from different populations do not recognize each other as potential mates, or are less attracted to each other. This can happen if mate preferences or other traits connected to sexual selection have diverged between populations as a result of ecological selection or processes like mutation-order speciation (Kirkpatrick and Ryan 1991, Mendelson *et al.* 2014, Servedio and Boughman 2017, Rundle and Rowe 2018). Ecological selection can lead to behavioural isolation through “magic traits” with ecological importance that also influence the mate choice of the species (Jiggins 2008, Servedio *et al.* 2011), or if the importance of sexual signals and the intensity of sexual conflict depend on ecological context (Rowe *et al.* 1994). In sympatric or parapatric populations, behavioural isolation can arise through ecological reinforcement (Rundle and Schluter 1998, Schwartz *et al.* 2010, but see Raeymaekers *et al.* 2010). However, behavioural isolation can also occur in allopatry as a by-product of divergent ecology, as demonstrated by examples where behavioural isolation is found to be stronger between allopatric populations that have adapted to different ecological environments, in comparison to isolated populations with similar environments (McKinnon *et al.* 2004, Vines and Schluter 2006, Langerhans *et al.* 2007).

Sexual selection can also lead to *mechanical isolation*, which occurs when fertilization is inhibited by incompatibility of reproductive structures such as genitalia. These incompatibilities are often studied in the context of non-ecological mechanisms, such as male-female conflict or cryptid female choice. However, reproductive structures can still be predictably influenced by divergent ecological selection, possibly leading to stronger mechanical isolation between different ecotypes (Anderson and Langerhans 2015).

Gametic isolation can occur after mating if gametes of different populations do not successfully form a zygote. The role of ecology in the formation of gametic isolation has been studied very little, but in *Timema* stick insects female fecundity has been shown to be lower when mating with a foreign male from a different ecotype in comparison to mating between individuals from similar ecotypes (Nosil and Crespi 2006a), suggesting that ecological divergence could lead to gametic incompatibilities.

Postzygotic isolation

Intrinsic postzygotic isolation refers to sterility or reduced viability of hybrids caused by incompatibilities between parental genomes. These incompatibilities commonly develop following the Dobzhansky-Muller model (Dobzhansky 1934, Muller 1942, Dobzhansky 1950). According to the model, selection normally favours combinations of genes that work well together, but if alleles in different loci have evolved in different directions between populations, the hybrid genotype may contain new combinations that are selected against during development (Figure 2). This process can happen either through ecological selection, mutation-order speciation, or simply by genetic drift. The intrinsic incompatibilities also seem to appear more frequently in the heterogametic sex, a phenomenon referred to as Haldane's rule (Haldane 1922, Orr 1997). Intrinsic postzygotic isolation has been extensively studied, but rarely in the context of ecological speciation. Most often, hybrid inviability or sterility is shown to increase fairly linearly after a sufficient divergence time (Sasa *et al.* 1998, Lijtmaer *et al.* 2003). However, there are several studies implicating that divergent selection could influence the evolution of intrinsic isolation (Dettman *et al.* 2007, Lee *et al.* 2008, Soria-Carrasco *et al.* 2014). For instance, divergence in an ecologically relevant trait, body size, has been shown to correlate positively with intrinsic postzygotic isolation in fish (Bolnick *et al.* 2006). Still, as explained earlier, parallel phenotypes with different genomes under similar ecological regimes can also lead to genetic incompatibilities through mutation-order speciation (Mani and Clarke 1990). Furthermore, intrinsic postzygotic isolation also does not always seem to correlate with ecological divergence, such as in *Jaltomata* plant species, where the strength of intrinsic incompatibilities was best explained by genetic distance (Kostyun and Moyle 2017). With fairly few studies, the strength of the link between ecology and intrinsic postzygotic isolation is still unclear.

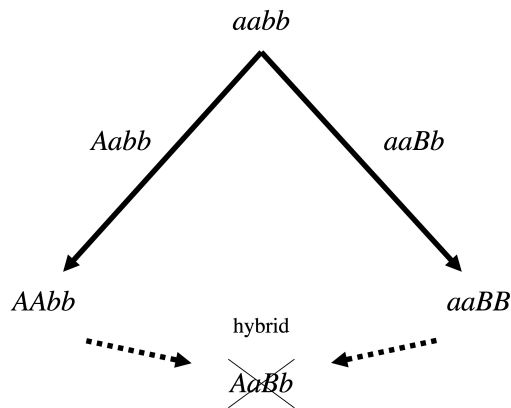


Figure 2. The Dobzhansky-Muller model (modified from Coyne and Orr 2004) explains how mutations appear and get fixed in two loci (A and B), leading to intrinsic reproductive isolation between the diverged populations. The incompatibilities between the loci appear only in the hybrid phenotype *AaBb*, which has never been tested by natural selection in the wild before hybridization.

Extrinsic postzygotic isolation refers to a hybrid phenotype being relatively unfit in the environment of its parents, as illustrated by the idea of hybrids falling into a fitness valley between parental fitness peaks (Figure 1). This “ecological hybrid inviability” is particularly connected to ecological speciation, as it can only develop under ecologically divergent selection (Rundle and Nosil 2005). For example, first-generation (F_1) hybrids between limnetic and benthic stickleback ecotypes show normal viability in laboratory conditions, but grow slower than their parents when confined to enclosures representing either of the parental environments in the wild (Hatfield and Schluter 1999). Such experiments still may not necessarily rule out intrinsic hybrid inviability from extrinsic, as it is possible that genetic incompatibilities do reduce hybrid performance, but this only becomes prominent under natural conditions (McFarlane *et al.* 2016). Studies have approached this problem in three ways: comparing the performance of hybrids to their backcrosses with parental populations, measuring hybrid performance also in an intermediate environment (if possible), or comparing hybrids between similar ecotypes to hybrids between different ecotypes. So far, all three approaches have provided evidence that hybrid inviability cannot be explained with only genetic incompatibilities. In sticklebacks, hybrid backcrosses with the limnetic ecotype show higher growth rates in open waters, and hybrid backcrosses with the benthic ecotype perform better in the littoral zone (Rundle 2002). In sagebrush shrubs, hybrids perform better than either of the parental subspecies (one adapted to basin, one to mountain environment) when grown at intermediate elevation (Wang *et al.* 1997). Finally, hybrids between two lake-adapted cichlid species seem to survive better in the lake habitat than hybrids between lake-adapted and river-adapted species (Rajkov *et al.*

2018). As demonstrated by Wang *et al.* (1997), hybrids are thus not necessarily inviable if there is an environment where the intermediate phenotype is beneficial. Hybrids fitting into intermediate niches may explain why, in the stickleback system, free-ranging wild hybrids do not seem to grow slower than the parental ecotypes under varying natural conditions, despite showing lower performance in experimental trials (Taylor *et al.* 2012).

A further form of reproductive isolation is *sexual selection against hybrids*, where hybrids fail to acquire a mate. This barrier can be affected by ecological divergence if ecologically relevant traits are also important for mate choice, and thus the traits of hybrids are unattractive. For example, hybrids between mimic species of *Heliconius* butterflies have colour patterns that do not fit parental mate preferences (Naisbit *et al.* 2001), substantially reducing hybrid fitness.

Relative importance of reproductive barriers

Once the speciation process has started, it is not guaranteed to stay in effect until the end result of two distinct species. In introgressive hybridization, gene exchange resumes between species or diverging populations, which may lead to speciation reversal. In other words, if populations have secondary contact before sufficient reproductive isolation has accumulated, newly diverged species may merge back into one hybrid swarm (Seehausen *et al.* 2008). The leading cause of recent introgression seems to be human-induced environmental change (Smith 1964, Lehman *et al.* 1991, Seehausen *et al.* 1997), which may have deteriorated the effect of some reproductive barriers. For example, the ecologically distinct limnetic and benthic morphs of sticklebacks have been shown to merge back into intermediate hybrids in areas with introduced crayfish, possibly because the consequent increase in water turbidity made the cost of mate choice higher (Taylor *et al.* 2006, Candolin *et al.* 2007). Although these hybridization events can also lead to birth of new hybrid species (Abbott *et al.* 2013) or act as an evolutionary rescue that provides needed genetic variation for the species (Oziolor *et al.* 2019), the genetic admixture of existing species is currently contributing to the ongoing loss of biodiversity around the world (Seehausen *et al.* 2008). In order to estimate the likelihood and potential effects of hybridization in different systems, we need more information on which reproductive barriers typically evolve first, and how much each barrier contributes to the overall strength of isolation.

Isolation consisting of multiple reproductive barriers is expected to be more stable (Coyne and Orr 2004), as single barriers are prone to leaking or disappearing more easily with environmental changes. Indeed, it seems to be fairly common that reproductive isolation involves more than one barrier simultaneously, and recent studies have started to estimate reproductive isolation as a sum of all measurable barriers

in that system (Lowry *et al.* 2008, Matsubayashi and Katakura 2009, Lackey and Boughman 2017, Rometsch *et al.* 2020, Chin and Cristescu 2021). Although all reproductive barriers are relatively common across taxa (Nosil 2012), some barriers are still considered to contribute more to speciation than others. From an individual's perspective, reproductive barriers take place sequentially. Regardless of hybrid viability, hybridization can only happen if the preceding isolating barriers have been cleared: two individuals of different sexes have to have met in the same environment, accepted each other as mates, had successful sperm transfer, *et cetera*. Because of this, the barriers that come into effect earlier (prezygotic barriers) are traditionally considered to be more important for restricting gene flow. However, conclusive evidence that prezygotic barriers would be the first important isolating barriers to appear has been lacking (Coyne and Orr 2004, Matute and Cooper 2021), partially because we can normally only study single species pairs at certain stages of speciation, and thus it is often difficult to tell which barrier has come into effect first.

Studies comparing how different barriers contribute to the total reproductive isolation usually find the impact of prezygotic isolation to be considerably stronger and accumulate faster than postzygotic isolation (Lowry *et al.* 2008, Stelkens *et al.* 2010, Lackey and Boughman 2017). Especially behavioural isolation is consistently one of the strongest measured barriers in animals (Matsubayashi and Katakura 2009, Nanda and Singh 2012, Martin and Mendelson 2016, Nava-Bolaños *et al.* 2017). Still, as demonstrated in a recent study by Irwin (2020), behavioural isolation could be too weak to restrict gene flow on its own in cases such as hybrid zones, and some form of postzygotic isolation would also be needed. The strength of ecological (habitat/temporal) isolation on the other hand seems to depend on the system: for example, it was the weakest measured barrier in darters (Martin and Mendelson 2016), but the strongest form of isolation in sticklebacks (Lackey and Boughman 2017), *Phytophagous* beetles (Matsubayashi and Katakura 2009) and some *Daphnia* species pairs (Chin and Cristescu 2021). Out of the different postzygotic barriers, ecological hybrid inviability is expected to have a significant effect earlier on the speciation continuum compared to intrinsic hybrid inviability (Seehausen *et al.* 2014). This is because genetic incompatibilities are expected to develop slowly over time, but hybrids can face ecological selection as long as the parental species are adaptively diverged. Clear exceptions do exist, such as how genome-wide duplication can lead to strong intrinsic reproductive isolation over a single generation (Levin 1983, Husband *et al.* 2016), but this is quite rare outside the plant kingdom. Still, it has recently been questioned if the effect of intrinsic hybrid inviability has been underestimated in studies of the early stage of speciation (Coughlan and Matute 2020). While there is some support for ecological hybrid inviability developing earlier in natural systems (Lackey and Boughman 2017), few studies compare the intrinsic and extrinsic forms of postzygotic isolation directly. In general, more studies comparing the strength of multiple forms of reproductive isolation at once would be needed in different species groups. For example, based on

pairwise comparisons between stickleback species with varying times since divergence, Lackey and Boughman (2017) suggest that habitat isolation is the first main contributor of emergence of reproductive isolation in this system, with behavioural isolation following soon as a strong secondary barrier, and postzygotic barriers evolve later with ecological hybrid inviability evolving first and any intrinsic isolation appearing last.

The extent to which ecological divergence contributes to each form of reproductive isolation is still unclear. While ecological selection is nearly always involved in immigrant inviability and ecological hybrid inviability, and has been shown to affect habitat, temporal and behavioural isolation, there has not been enough research to conclude if it is commonly involved in gametic isolation, intrinsic hybrid inviability or sexual selection against hybrids (Rundle and Nosil 2005). It has also been estimated that ecological divergence is associated more with prezygotic isolation rather than postzygotic isolation, but this estimation does not make further distinctions between the different barriers (Funk *et al.* 2006). Isolating barriers that rely on ecological divergence can lead to particularly rapid speciation (Momigliano *et al.* 2017), but they may also be prone to weakening if the original ecological regimes merge or disappear. For example, if hybrid inviability is only ecological, environmental change can create an intermediate environment in which the hybrid phenotype thrives (Wang *et al.* 1997). This may result in the collapse of a species pair or a new separate hybrid species (Seehausen *et al.* 2008). Extrinsic hybrid viability can also fluctuate spatially and temporally (Arnold and Martin 2010), which can lead to partial reproductive isolation as an adaptive optimum (Servedio and Hermisson 2020), where hybrids should still remain in the minority as long as the selection against them is sufficient on the yearly average. Homogenisation of the environment may also similarly weaken ecological isolation and immigrant inviability, as well as behavioural isolation (Seehausen *et al.* 1997). The only reproductive barriers that could strongly resist reversal are mechanical isolation, gametic isolation and intrinsic hybrid inviability, as they are dependent on individual genome and morphology, and thus should remain regardless of abrupt environmental change. As these types of isolation are also the least studied in terms of ecological speciation, additional research could give us a better idea of whether ecological selection can lead to irreversible speciation on its own, or if reproductive isolation persists only in the original ecological context until the later stages of the speciation continuum.

Unresolved issues

Although many reproductive barriers have been studied extensively, some barriers have received less focus, especially in the context of ecological divergence. The relative contribution of different reproductive barriers to the overall reproductive isolation has still been compared only in a few systems, but especially comparative studies across varying levels of ecological divergence are critically needed (Nosil 2012). As the strength of multiple reproductive barriers are estimated in more taxa at different stages of speciation, we are able to better estimate which barriers evolve faster and how they are affected by divergent ecology. Ecological divergence has also been studied most often in the context of abiotic differences or specialisation to a certain food source or host, likely because they are easily quantifiable and expected to act as a relatively stable source of selection. However, interactions within and between species, such as competition and predation, are potential sources of selection that have been understudied in the context of ecological speciation.

It is also unclear to which extent divergent selection can drive reproductive barriers in different geographical contexts. Most studies comparing the relative strength of isolating barriers in a single system include species pairs with a varying level of geographical isolation, but it is rarely considered which barriers can evolve in full allopatry. A study with *Timema* stick insects by Nosil (2007) estimates that habitat preferences and immigrant inviability act as stronger isolating barriers in allopatric species pairs, while behavioural isolation had a stronger effect in sympatric populations. Thus, different reproductive barriers may be more important for speciation depending on whether they have evolved as a by-product of selection, or through reinforcement-like processes. In order to disentangle these effects, similar comparisons would be required in systems where reinforcement cannot exist, such as allopatric populations without secondary contact.

Predation as a source of ecological selection

Predation has likely existed almost as long as there have been different living organisms on the planet. For the prey, successful predation will result in the ultimate fitness cost, death. This fact alone makes predation a strong source of natural selection, so it is not surprising that evolution has led to a large variety of morphological and behavioural traits that help prey avoid or escape predation (Ydenberg and Dill 1986, Young *et al.* 2004, Eklöv and Svanbäck 2006, Dingemanse *et al.* 2007, Lapiedra *et al.* 2018). Yet, the threat of predation is not constant everywhere. Many environments can be divided into high-risk and low-risk habitats (Gaynor *et al.* 2019) and major predators can be missing entirely on islands or in isolated lakes. Variation in predation risk can thus act as a source of divergent selection which may lead to adaptive radiation in prey. As an

example, the different colour morphs of stick insects face diverging selection only under high predation risk, since individuals not matching their host plant are more vulnerable to predators (Nosil and Crespi 2006b). As human impact often leads to fragmentation and isolation of environments, the connectivity between low-risk and high-risk habitats may become weaker in the future (Boone and Hobbs 2004, Araujo *et al.* 2014). Furthermore, the anthropogenic effects have resulted in diminishing numbers of top predators in both terrestrial and aquatic ecosystems across the world (Myers and Worm 2003, Strong and Frank 2010, Estes *et al.* 2011), but also introduced predators into areas where there were none before (Remeš *et al.* 2012). Thus, in order to fully realize the major top-down effects this variation in predation risk can have on entire ecosystems, it is essential to further our knowledge of how varying levels of predation pressure may drive the evolution of prey. However, quantifying the differences in predation pressure in the wild or manipulating predation risk in captivity is often challenging. This may also explain why in most studies examining ecological speciation, the divergent natural selection typically originates from resource use or abiotic environmental differences (Rundle and Nosil 2005). Below, I review the few studies that have examined the role of predation on different reproductive barriers, further identifying gaps in our current knowledge.

Most prezygotic barriers have been studied fairly little in the context of predation risk, but a few exceptions exist. Divergent adaptation to two interconnected habitats with different levels of predation pressure may lead to habitat isolation between different ecological phenotypes, as shown in *Asellus* isopods (Eroukhmanoff *et al.* 2011). *Brachyrhaphis* fish adapted to a low-predation environment have been shown to have lower survival in a high-predation environment, so immigrant inviability can be a strong reproductive barrier between populations adapted to different levels of predation (Ingle and Johnson 2016). Mate choice, and thus behavioural isolation, may also be affected by predation in several ways. First, many sexual signals cause the signaller to be more susceptible to predators, and high predation risk can therefore reduce the expression of morphological traits, courting behaviour or other signalling (Zuk and Kolluru 1998, Santema *et al.* 2019). Also, if a trait is preferred in mate choice because it predicts better survival, attractiveness of the trait may change depending on predation risk (Teyssier *et al.* 2014). Moreover, choosiness and mate searching time can leave the choosing individual at risk (Rowe 1994), so picking a mate quickly may be more beneficial if predation risk is high (Forsgren 1992). This can lead to weaker behavioural isolation, if females do not discriminate against foreign males as strongly in the presence of predators. This seems to be the case in túngara frogs, where females usually prefer conspecific calls, but under high predation risk they are more likely to approach a heterospecific male (Bonachea and Ryan 2011). The few studies that have directly studied the mate choice between phenotypes adapted to different predation levels, have found signs of predation risk driving behavioural isolation (Schwartz *et al.* 2010, Eroukhmanoff *et al.* 2011, Perini *et al.* 2020). However, in most of these studies the

level of predation risk covaries with several other environmental variables that define the specific habitats (up- vs. downstream, vegetation type, or amount of wave action), so the effects of these confounding variables often cannot be fully ruled out. More research on the effects of varying predation risk on prezygotic isolation is therefore needed, especially in systems where predation level is not connected to other ecological attributes of the environment.

Ecologically intermediate hybrids produced between populations adapted to high and low levels of predation would be expected to be more susceptible to predation than the parents adapted to high predation pressure. A few studies have found that hybrids are predated at a higher rate than their parental species, but since they have not found the mechanism linking hybrid phenotypes to higher predation risk, it is still unclear if this is caused by the ecological performance or some form of intrinsic inviability of the hybrids (Casas *et al.* 2012, Melo *et al.* 2014, Nilsson *et al.* 2017). A recent experiment studied the fitness of two mosquito species, one breeding in small rainfall-based pools and the other breeding in larger waterbodies with more predators, as well as their hybrids, raised with either no predators or with predatory backswimmers (Niang *et al.* 2020). While the parental species showed predictable mortality differences, one outcompeting the other in the no-predation environment and the other persevering more often in high-predation environment, the hybrid mortality was usually not intermediate, but rather had similar fitness to their mother's species. The study only identified female mosquitos, so whether the same applies to males remains to be seen. In species with mimicry however, hybrids with unsuccessful mimicry have been shown to suffer from higher predation rate (Jiggins 2008, Pfennig *et al.* 2015). With only a few studies so far, more examples from both laboratory experiments and nature would help confirm if predation can lead to ecological hybrid inviability in other systems as well.

Study systems

In my thesis work, I study the effect of predation on reproductive isolation in two systems. The majority of my work focuses on the multiple allopatric populations of Bahamas mosquitofish (*Gambusia hubbsi*), which face divergent predation pressures depending on the presence/absence of the predatory fish bigmouth sleeper (*Gobiomorus dormitor*). In addition, I examine the fitness of naturally occurring hybrids between a sympatric species pair of two cyprinids, common roach (*Rutilus rutilus*) and bream (*Abramis brama*), which face predation from great cormorants (*Phalacrocorax carbo*) in their native lake.

Bahamas mosquitofish

The Bahamas mosquitofish is a live-bearing fish (Figure 3) with over 20 allopatric populations in inland blue holes on Andros island, the Bahamas (Figure 4). Blue holes are collapsed former caves that have been colonized by fish at the time ocean levels rose after the last interglacial period ~15,000 years ago (Fairbanks 1989, Mylroie *et al.* 1995), but today are without in- or outlets, except for some underground tunnels filled with anoxic water (Bottrell *et al.* 1991). These small but often vertically deep lakes are simple, stable environments with typically only 1-3 fish species. The mosquitofish populations inhabiting the upper freshwater lens of these blue holes are highly isolated. These populations show some of the highest F_{st} values measured in fish and appear to be independent sinks of the original source population (Schug *et al.* 1998). Thus, the mosquitofish system provides a “natural experiment” with multiple populations with low gene flow at the same stage of early speciation.

Some of the blue holes have also been colonized by a major predatory fish, the bigmouth sleeper, and the presence or absence of this species has created two divergent ecological regimes for the mosquitofish populations. The high-predation regime, where the bigmouth sleepers are present, has high risk of predation and consequently lower mosquitofish population densities, whereas the opposite is true for the low-predation regime, which lacks any major predators (Langerhans *et al.* 2007, Heinen *et al.* 2013, Langerhans 2018). The blue hole system provides an exceptional opportunity to study the effects of predation apart of other confounding factors, as the level of predation risk does not covary with other major environmental variables or the genetic relatedness of the mosquitofish populations (Langerhans *et al.* 2007, Heinen *et al.* 2013, Riesch *et al.* 2013). Bahamas mosquitofish show many adaptations that have diverged between the predation regimes. For example, mosquitofish from high-predation environment have a body shape optimized for fast-start swimming bursts that allow escape from predators, whereas low-predation fish have a body shape better suited for energy-efficient prolonged swimming (Langerhans *et al.* 2007, Langerhans 2009a, b, Araujo *et al.*

2017). Thus, high-predation fish also show higher survival in predation trials (Langerhans 2009a), and fish with a body shape similar to the low-predation phenotype show higher growth rates (Araujo *et al.* 2017). Other examples of mosquitofish traits that have diverged between predation regimes include life-history (Riesch *et al.* 2013), antipredator behaviours (Fowler *et al.* 2018), sociability and exploration behaviours (Heinen-Kay *et al.* 2016), as well as stress physiology (Vinterstare *et al.* 2021).



Figure 3. Illustration of female (above) and male (below) Bahamas mosquitofish.

The mosquitofish populations represent a stage of ecological speciation where gene flow has relatively recently been limited by a geographical barrier, but where the populations under divergent natural selection have not yet had secondary contact. Since any hybridization between the populations is rare, reproductive isolation could only evolve to be stronger between different predation regimes as a by-product of divergent ecological selection. While the level of overall reproductive isolation between Bahamas mosquitofish populations has not yet been estimated, signs of ongoing ecological speciation have already been identified. The body shape and dorsal fin colour in males are predator-mediated morphological traits that also seem to matter in mate choice, which may result in behavioural isolation between predation regimes (Martin *et al.* 2014, Heinen-Kay *et al.* 2015). Indeed, when presented a choice between videos of two males, one native and one foreign, female mosquitofish showed stronger preference for their native males when the foreign male was from an opposite predator regime (Langerhans *et al.* 2007). The direction of morphological changes in both the male (gonopodium) and female (gonopore) genitalia have also diverged between predation

regimes (Anderson and Langerhans 2015). This suggests that behavioural and mechanical isolation are major potential contributors to ecologically mediated reproductive isolation between these populations. In a possible future secondary contact, immigrants and hybrids produced between predator regimes would also be likely to perform poorly in either environment, with higher vulnerability to predation in high-predation regime and lower competitive ability in low-predation regime. On the other hand, as the populations have been isolated for only a short time from an evolutionary standpoint, intrinsic incompatibilities are expected to be comparatively weak (Bolnick and Near 2005).

As *Gambusia* populations are fairly small, founder effects and drift may also affect the direction of their evolution. However, since each predation regime has several replicates, it is highly unlikely that these effects alone could cause evolution to move to the same direction in every population of the same predation regime. Thus, this system also provides a way to compare the effects of ecological selection and drift as major drivers of reproductive isolation.

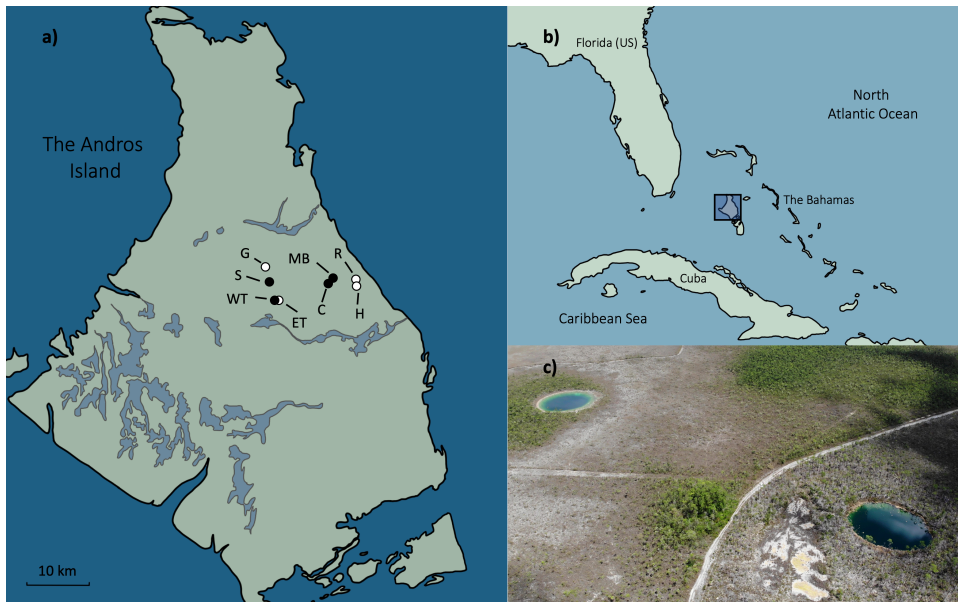


Figure 4. (a) Locations of the blue holes studied in this thesis. White marks annotate a low-predation blue hole (G= Gollum, ET = East Twin, R = Rainbow, H = Hubcap) and black marks annotate a high-predation blue hole (S = Stalactite, WT = West Twin, C = Cousteau's, MB = Murky Brown). (b) Location of the Andros Island. (c) West Twin and East Twin from air.



Figure 5. Example individuals of two bream (on the left), two roach (on the right) and a morphologically intermediate roach \times bream hybrid (in the middle).

Roach, bream and their hybrids

Roach and common bream are common cyprinid freshwater fish with overlapping reproduction habitats and seasons. Hybridization between roach and bream is fairly common (Figure 5), but the resulting intermediate roach \times bream hybrids are rarely found in great numbers. Thus, this species pair represents a stage of speciation where secondary contact has occurred and while some gene flow exists between the species, reproductive isolation seems to keep the species separate. Hybrids between roach and bream show asymmetrically lower fertility (Wood and Jordan 1987), but are still capable of backcrossing to parental species and are even relatively common in areas where species have recently been introduced (Toscano *et al.* 2010). Roach \times bream hybrids seem to be more exposed to predation by cormorants (Nilsson *et al.* 2017), but the mechanisms behind this higher predation risk are still unclear.

Both roach and bream show seasonal, partial migration, with part of the population migrating from their native lake to connected streams for the winter season (Skov *et al.* 2008). The timing of this seasonal migration depends on temperature-driven changes in the trade-off between predation risk and growth potential (Brönmark *et al.* 2008): during summer both the risk of predation and availability of food are higher in the lake, but during winter, resources in the lake diminish closer to the level of the stream habitat. Thus, moving to the stream habitat during the cold season is beneficial especially for individuals vulnerable to predation (Skov *et al.* 2011) and it has been shown that individuals with a higher perceived predation risk choose to migrate more

commonly (Hulthén *et al.* 2015). Individual predation risk in freshwater fish is typically affected by predator gape-size limitations. Bream, with a deeper body shape than roach, thus typically escape predation risk after they reach a certain size. The F_1 roach \times bream hybrids seem to be morphologically intermediate (Nilsson *et al.* 2017), so their higher vulnerability to predation might be caused by maladaptive behaviour instead. As migration behaviour of both roach and bream is connected to predation risk, any deviant behaviour exhibited by their hybrids may expose them to predators.

Aims of the thesis

The aim of this thesis is to identify how ecological selection in the form of predation can affect different types of reproductive barriers. Following the ecological speciation hypothesis, I expect differences in predation pressures to lead to divergent adaptations, which in turn will lead to stronger isolation between populations (**papers I-III**). In addition, I expect predation to act as a strong selective agent against maladapted hybrids (**papers III-IV**).

In order to compare the effects of differences in natural selection between two environments, we need to identify which traits are under positive selection in environment A, and which are selected for in environment B. In **paper I**, I aim to fill the gap in the knowledge about the selection acting in environments that lack major predators. I expect that in the absence of predators, higher intraspecific densities will create an environment that selects for highly competitive individuals. If the results support my hypothesis, similar methods can further be used for testing the ecological performance of hybrids in **paper III**. In addition, the results allow us to estimate the strength of immigrant inviability of the hypothetical migrants from the high-predation to the low-predation environment.

In **papers II-III**, I experimentally create mating pairs between several allopatric mosquitofish populations, which allows me to identify which forms of reproductive isolation may have evolved as a by-product of divergent ecological selection. **Paper II** focuses on behavioural isolation, while **paper III** examines hybridization success as well as intrinsic and extrinsic hybrid inviability. Based on the strongly predictable ecological adaptations in this system, I predicted both behavioural isolation and ecological hybrid inviability to be stronger between individuals from different predation regimes. As a result of ecology-mediated prezygotic barriers, I similarly expected hybridization success to be lower in mating pairs between different environmental regimes. Additionally, as the populations have diverged very recently, I predicted intrinsic hybrid inviability to be weak. However, in case we found evidence of intrinsic incompatibilities, I expected that they would be most prominent in hybrids generated between different predation regimes.

The allopatric nature of the mosquitofish system prevents us from observing selection on hybrids directly in the natural environment. Thus, in **paper IV** I examine another natural system with the sympatric roach, common bream and their hybrids. The aim

of this study is to observe if the migration behaviour of hybrids differs from the parental species, and whether this leads to higher predation pressure on hybrids. I expect the migration behaviour of hybrids to differ from both parental species, and that this migration behaviour links to higher predation risk in hybrids. This would be an example of predator-mediated extrinsic hybrid inviability in the wild, which currently has only a few documented examples.

Methods

In **paper I**, I studied differences in foraging behaviour in wild mosquitofish from high- and low-predation environments in a standardized environment free of predation risk. I expected individuals from low-predation populations to forage more actively and efficiently than high-predation individuals.

Foraging trials were used to evaluate the feeding behaviour of individuals from six (three high-predation and three low-predation) mosquitofish populations. Fish were caught in the wild (Figure 6) and then acclimated to laboratory conditions for at least one day before the start of the trials. Fish were fed in the evenings and starved overnight to standardize hunger levels among individuals. Two adult fish of the same sex were placed in a translucent arena filled with water from their own blue hole and left to acclimate for 15 minutes. At the start of the trial, 15 small (1-2 mm) pieces of Chironomid larvae were added to the tank. Each trial was filmed and foraging behaviour was recorded from the videos for the first 15 minutes of the trial. Using the Behavioral Observation Research Interactive Software BORIS (Friard and Gamba 2016), I manually recorded each foraging attempt (defined as an individual's mouth contacting or distinctly nudging towards the bottom of the tank) and each successful food consumption event, which were used as proxy for foraging rate and food consumption rate respectively. I also calculated foraging efficiency as the proportion of successful foraging instances out of all foraging attempts. The foraging rate, food consumption rate and foraging efficiency were then compared between the different predation regimes.



Figure 6. a) Field collection at Cousteau's blue hole. b) Foraging trials conducted at the field laboratory.

In **paper II**, the aim was to use one-on-one mating trials to investigate if mating behaviours of Bahamas mosquitofish had diverged between predation regimes, and to compare the level of behavioural isolation between populations adapted to either similar or different predation regimes. I expected that especially females would prefer mates from their own population the most, followed by foreign individuals from a similar predation regime, while showing least preference for males from a different predation regime.

Juvenile Bahamas mosquitofish had been collected from eight populations in the wild (4 high-predation, 4 low-predation, Figure 4) and transported to laboratory facilities at North Carolina State University in 2016. These fish were then reared to adulthood and mated in the laboratory, and their laboratory-born offspring were used in the mating trials after they had reached maturity. All eight populations were matched up with a planned matrix of 56 inter-population combinations with ~6 replicates each. However, the population from the Murky Brown blue hole did not produce enough offspring for all mating combinations, so it could not be used for most inter-population matings. As we had to drop Murky Brown from the setup, we also decided to exclude the low-predation site with the least performed trials at the time (Gollum) from the mating behaviour analyses in order to keep the population matrix balanced. However, both were still used to test whether within-population mating behaviours had diverged between predation regimes.

Before mating trials, the adult mosquitofish were moved into individual experimental tanks in the evening and left to acclimate overnight. At the start of each trial, a male was quickly moved into the adjacent female tank and their interactions were filmed for the following 20 minutes. All distinct mating behaviours were manually recorded from

the videos using the BORIS program. First, we investigated whether either female or male mating behaviours predictably diverged between predation regimes in within-population trials. Secondly, we focused on key behavioural variables, including the number of male lateral displays, male close approaches, male forced copulation attempts, female avoidance responses and female aggression towards the male. The expression of each of these behaviours were compared between three different mate types: native mate from the same population, foreign mate from the same predation regime, and foreign mate from a different predation regime.

After the trial, a plastic plant was added into the tank to reduce stress from potential harmful interactions, and the pair was left together for ~22 more hours before being separated. Each fish was weighed and photographed with a standardized scale. Females were then moved into individual small tanks and monitored daily for 110 days after mating. Any offspring born to the females were photographed and moved into a separate tank upon discovery. After the monitoring period, the females were sacrificed and preserved, as well as x-rayed for any remaining fertilized embryos. Most of the hybrid offspring were reared to adulthood and tested as a part of **paper III**. As we did not acquire enough hybrids from these trials alone, additional mating tanks were set up where the female and male were allowed to interact for several days before being separated. In both types of mating pairs, the females were also observed for possible aborted developing embryos, and after the 110-day monitoring period had ended, the females were further x-rayed for possible developing embryos. The number of trials that successfully resulted in hybrid zygotes was used for comparing the hybridization success between within-regime and between-regime mating pairs in **paper III**.

Paper III examined both intrinsic and extrinsic viability of hybrids produced within and across predation regimes. Due to the fairly recent divergence of mosquitofish populations, I expected intrinsic inviability to be minimal, but in case some hybrids had lower viability, I predicted that hybrids produced between different predation regimes would be the least viable. Furthermore, I expected that hybrids between different predation regimes would show intermediate performance in key traits predicting high fitness in either high-predation or low-predation regime, while hybrids produced within the same predation regime would perform similarly to their parents.

Hybrids and pure-line offspring were photographed at birth and reared under the same laboratory conditions. Due to space limitations, similarly aged offspring within the same population combination were pooled into the same tank. At approximately 6 months of age, the surviving fish were sexed, counted and photographed. The fish were split into same-sex tanks with 5-6 individuals each, and reared until 10 months of age, by when they were counted again and used in ecological performance trials. Offspring survival was measured as number of surviving individuals at 6 and 10 months of age.

The hybrids and pure-line offspring were tested in two key ecological performance traits, one representing performance in high-predation environments, and the other reflecting performance in low-predation (and, thus, high-competition) environments. Fast-start is a typical escape response where the fish bends its body into a C-shape before rapid acceleration of movement. Better fast-start performance has been shown to predict higher survival with predators in mosquitofish (Langerhans 2009a). The other key performance trait was measured as foraging rate and food consumption rate. Fish were transferred to individual experimental tanks and left to acclimate overnight before trials. Foraging trials started with 10 small Chironomid larvae pieces added into the tank, after which the trial was filmed for 6.5 minutes. Foraging and food consumption rates were extracted from videos in the same way as in **paper I**.

Before each fast-start trial, the fish individual was transferred from home tanks into an experimental arena with 30 mm of water to limit vertical movement. Fast-start responses were filmed with a high-speed video camera from underneath the arena using a mirror placed below the arena inclined at 45 degrees. Each subject was allowed to settle for one minute before it was startled by waving a hand over the arena. I picked the most high-quality recorded fast-start for each individual, and further analysed the first 30 frames of the response using the DLTdv8a tracking software (Hedrick 2008). The performance variables recorded were rotational velocity (i.e. velocity of the full body of the fish curving into a maximum C-shape), maximum acceleration of the centre of mass and total distance moved by the centre of mass.

In **paper IV**, I examined the seasonal migration behaviour of roach, bream and their hybrids using electronic tags and passive telemetry, which allowed me to connect individual migratory behaviour to individual predation events. I predicted that hybrids would show intermediate or novel migration behaviour when compared to roach and bream, and this might leave them vulnerable to predation.

The migration data for roach, bream and their hybrids were collected in 2005-2006 at the Danish lake Loldrup Sø. The fish were caught by electrofishing, weighed and a Passive Integrated Transponder (PIT) tag was implanted into their body cavity before they were released back into the wild before the migration period. Both the inlet and outlet of the lake had antennas that recorded each PIT-tag detection with the time and identity code of the fish. After natural predation had had time to occur, the ground below the nearby colonies and roosts of great cormorants were scanned with a PIT-tag detector multiple times over several months in 2007-2008. This allowed us to identify individuals that had been eaten by a cormorant, as the tags are regurgitated and fall to the ground after consumption.

I compared the migration behaviour between roach, bream and roach \times bream hybrids in two variables, a) migration frequency (number of migration trips between lake and stream) during the October-December period and b) timing of initiation of migration

(date of the first trip from the lake to the stream). I also analysed whether migration frequency or timing of migration initiation predicted whether the fish was eaten by cormorants. Since individuals predated during the season would naturally have less migration trips, for the predation analysis I adjusted the migration frequency to the observation period (days from first detection to last detection) for the individuals that were not confirmed to be alive (i.e. did not show any activity) the next spring.

Main results

Traits under selection in low-predation regimes

In **paper I**, I found that fish from different predation regimes differed in foraging rates even under standardized conditions with no risk of predation, but this effect was sex-specific. Female mosquitofish showed a higher foraging rate and consumed more food if they originated from populations that had adapted to conditions with no major predators, as opposed to females adapted to high predation risk (Figure 7 a-b). However, this difference between predation regimes was weaker in male mosquitofish and had an opposite trend from females, as males from high-predation sites had slightly higher foraging rates than males from low-predation populations.

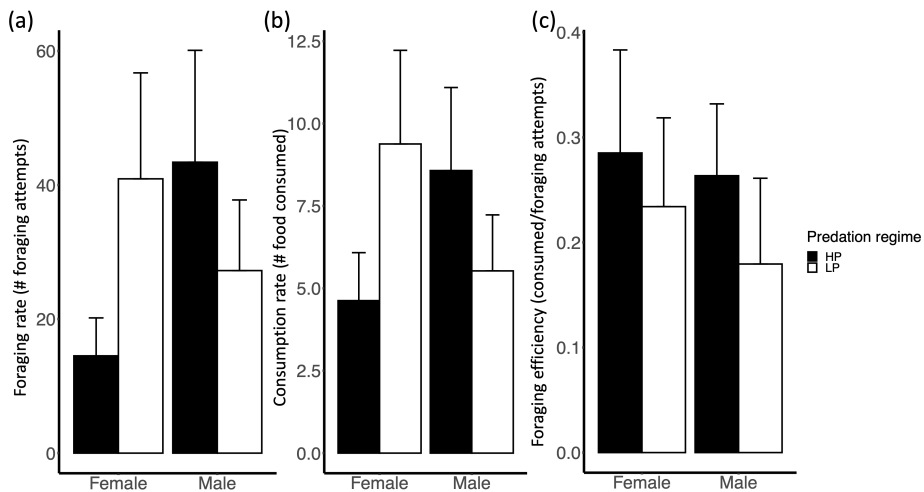


Figure 7. Variation among sexes and predation regimes in (a) foraging rate, (b) consumption rate and (c) foraging efficiency. Values depict estimated marginal means and error bars denote standard error. HP = high predation, LP = low predation.

These results support the hypothesis that environments with low predation risk can select for highly competitive individuals that are active at foraging, at least in females. Since female mosquitofish give birth to live young and are significantly larger than males in body size, they may simultaneously have a higher need for acquiring resources, and experience a higher predation risk in the wild. This stronger trade-off between different levels of predation risk could explain why I was only able to detect strong differences in female foraging behaviour. Males, on the other hand, may value mating opportunities more than foraging, so they may have adjusted their behaviour to forage more in the context of my experiment, where neither females or predators were nearby.

Selection for traits associated with high competitive potential in low-predation environments could potentially contribute to reproductive isolation in two ways. First, immigrants from high-predation environments could be outcompeted in low-predation lakes, leading to some level of immigrant inviability. Second, hybrid offspring between low-predation and high-predation individuals may have lower fitness if they are outcompeted by the native fish. I will return to this question in **paper III**.

Behavioural isolation

No-choice mating trials revealed differences between predation regimes in mating behaviour (**paper II**). Male mosquitofish differed between regimes in overall mating behaviour, with high-predation males displaying higher courting activity and higher number of mating attempts than low-predation males. However, male mating behaviour did not significantly change depending on the origin of the female they were matched with. On the other hand, females did not show differences in their behaviour between predation regimes, but did respond to males differently depending on the male origin. More specifically, females showed higher aggression levels when matched with a foreign male, and initiated aggression fastest with foreign males originating from the opposite predation regime (Figure 8). As male coercion is very common in mosquitofish, females seem to rarely display receptive mating behaviour, and instead may assert mate choice through resistance to unwanted matings. The large body size of the females can make them a serious threat to males, so female-to-male aggression may be an effective way to reject unwanted males in the wild. The females thus showed signs of ecologically mediated behavioural isolation by initiating aggression later on males from the same predation regime, whether they were native or from a foreign population.

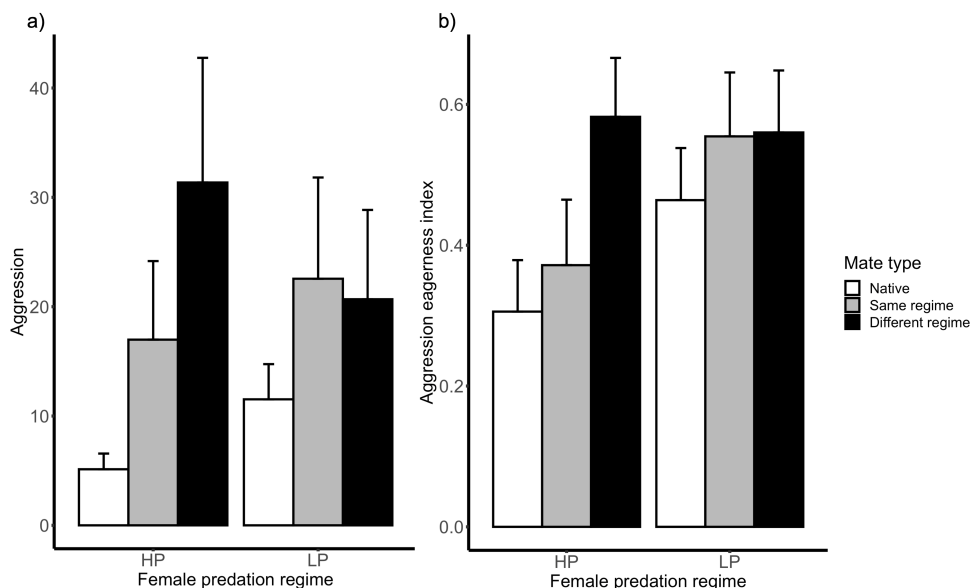


Figure 8. The effect of mate nativity and predation regime (native / foreign: same regime / foreign: different regime) on a) number of instances of female aggression and b) eagerness of female aggression (higher index value indicates faster initiation of aggression).

Hybridization success

Mating pairs within the same predation regime had a significantly higher hybridization success, when compared to pairs between different predation regimes. As successful fertilization was determined to have happened if the female gave birth to any live or stillborn offspring, or if x-rays revealed fertilized embryos inside the female at the end of the observation period, the isolating barriers inhibiting successful hybridization were likely prezygotic. Both behavioural isolation and mechanical isolation are especially likely contributors to unsuccessful hybridization in this system. This further enforces that differences mediated by the predation regime have led to significantly stronger prezygotic isolation.

Intrinsic hybrid inviability

The hybrids reared in common garden laboratory facilities for **paper III** showed different mortality levels depending on the ecological adaptations of their parents. Hybrid offspring originating from parents mated across different predation regimes had the highest mortality compared to native offspring, while hybrid crosses within the same predation regime showed intermediate mortality (Figure 9 a,b). This result shows that divergent ecological adaptation can generate more intrinsic incompatibilities between populations, thus leading to higher intrinsic hybrid inviability. Assessing the sex of the hybrid offspring also revealed that the higher hybrid mortality was only prominent in females, while male hybrids had similar mortality levels to native offspring (Figure 9 c).

Signs of hybrid sterility or inviability often show first in only one sex, typically in the heterogametic sex, as stated by Haldane's rule (Haldane 1922, Orr 1997). While the sex determination mechanism of female Bahamas mosquitofish is still unconfirmed, Haldane's rule likely applies here, as females are heterogametic in some closely related species (Kottler *et al.* 2020), and Bahamas mosquitofish males do not have heteromorphic sex chromosomes according to our preliminary karyotype data (Langerhans 2021, unpublished). Regardless, the mosquitofish populations have only been isolated for ~15.000 years, so signs of higher hybrid mortality show that intrinsic hybrid inviability can emerge relatively early in the speciation process.

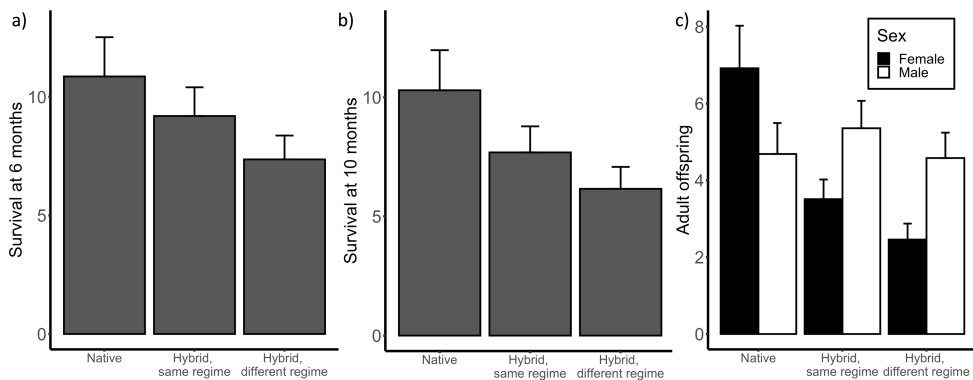


Figure 9. Estimated number of surviving offspring per cross type at a) 6 months, b) 10 months and c) adult (over 10 months) by sex.

Ecological hybrid inviability

I estimated ecological viability of hybrids through laboratory trials in **paper III** and through observations of fish migration behaviour in the wild in **paper IV**. In the laboratory trials, I measured the performance of hybrid mosquitofish in two ecologically important traits that should be closely linked to their fitness in the two natural predation regimes respectively. Following my predictions, hybrids produced between predation regimes showed intermediate performance in fast-start ability, a trait which has been shown to predict survival against predators. This indicates that hybrids born between native high-predation and migrant low-predation parents would suffer higher mortality from predation than native offspring, following the principle of ecological hybrid inviability. However, I could not detect similar differences in foraging behaviour, as laboratory-raised fish also showed little differences between predation regimes. Fast-start performance is closely linked with the individual's body morphology, which might make the differences in fast-starts persist more easily in laboratory conditions, as opposed to foraging behaviour, which may be more flexible depending on context.

In **paper IV**, roach \times bream hybrids showed both intermediate and novel migration behaviour, depending on the trait. The date at which hybrids first initiated migration to connecting streams was intermediate between roach and bream, which is in line with previously described intermediate migration behaviour in other systems (Moore *et al.* 2010, Delmore and Irwin 2014). Interestingly, the hybrids showed elevated frequency of migration trips between the lake and stream habitats compared to either parental species. Furthermore, the individuals that had a higher number of migration events during the season were shown to be at higher risk of cormorant predation (Figure 10). While we cannot confirm whether the behaviours expressed by hybrids are due to genetic effects or other reasons, it seems that the displayed novel behaviour is maladaptive and thus ecologically selected against. The observed multi-trip migration behaviour can come with costs such as higher energy expenditure (Brodersen *et al.* 2008), but most likely also makes the individual more likely to be detected by predators congregated around migratory corridors, especially if they are easily distinguished due to being unsynchronized with other migrating individuals (Harts *et al.* 2016). Predation may thus enforce species integrity by reducing survival of roach \times bream hybrids in the wild.

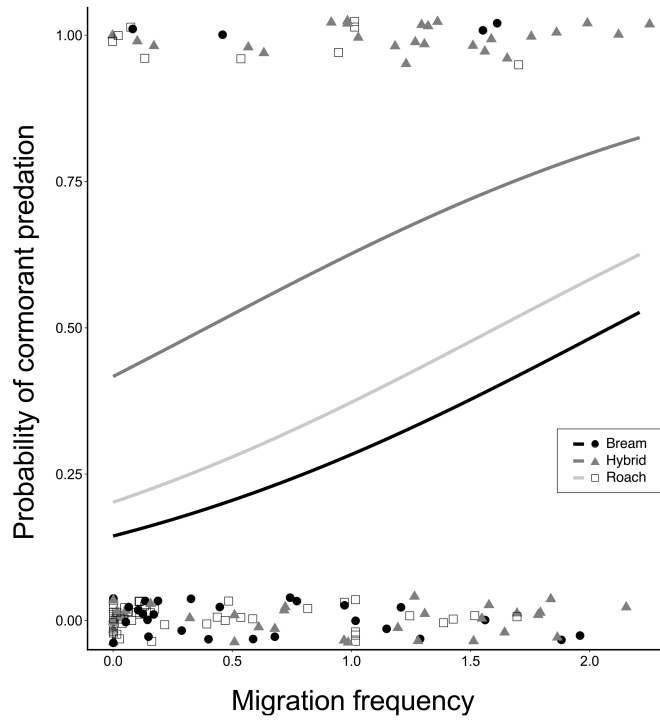


Figure 10. Effect of migration frequency on the probability of cormorant predation for bream, roach and their hybrids. Curves visualize probability distributions predicted by a GLM (binomial, logit-link) model on individual fish migration frequencies and fate (predated (1) or not (0)), denoted by jittered raw data points.

Unpublished results

Female aggression as a component of behavioural isolation

While **paper II** found that females were more aggressive towards males that originated from a different ecological regime, it did not study which cues the females may use as a basis for this behaviour. We studied whether differences in morphology, i.e. the body shape of the fish, could predict the aggressive female behaviour. Body shape predictably diverges between predation regimes, and the mating preferences of female mosquitofish have previously been shown to be influenced by how much the male differs from the female in ecologically driven body shape (Langerhans *et al.* 2007). After the mating trials, every fish was photographed from a lateral view. From the lateral photographs, 16 landmarks (Figure 11) representing the main body shape were marked for each individual using tpsDig (Rohlf 2015). The landmark coordinates were transferred into the MorphoJ program (Klingenberg 2011), where we performed a Procrustes fit on the coordinates in order to remove effects of position and size. Using the resulting partial warps, we conducted a discriminant function analysis (DFA), which tested how successfully the body shapes could be categorized between high-predation and low-predation regimes. The individual values from the DFA gave us a single axis ranging from low-predation to high-predation body morphology. Male and female data were handled together, as we wanted to estimate the differences in predation-driven morphological between the individual males and females, and thus they needed to be placed on the same morphological axis. Individual scores of the morphology axis were further analysed in R. For each mating pair, a morphological distance was calculated as the absolute distance between the male and female values. The effect of male body shape on the level of female aggression and initiation of aggression was evaluated with a generalized linear mixed model (GLMM). Both models included female regime and its interaction with morphological distance, female and male centroid size (as an estimate of body size), as well as female and male populations as random factors.

When the morphological distance between the male and the female was larger, the females were generally more aggressive ($\chi^2 = 5.74$; Df = 1; $p = 0.0166$; Figure 12 a) and initiated aggression earlier ($\chi^2 = 6.90$; Df = 1; $p = 0.0086$; Figure 12 b). None of the other factors or interactions in the model had significant effects. This fits our prediction that females adjust their aggression behaviour using visual cues of the male, and more

specifically reject males more easily when they have diverged from the ecological phenotype of the female. Our results further support that aggression could be an important mating behaviour and contribute to the predator-mediated behavioural isolation in this species.



Figure 11. Positions of the 16 morphological landmarks collected from each individual.

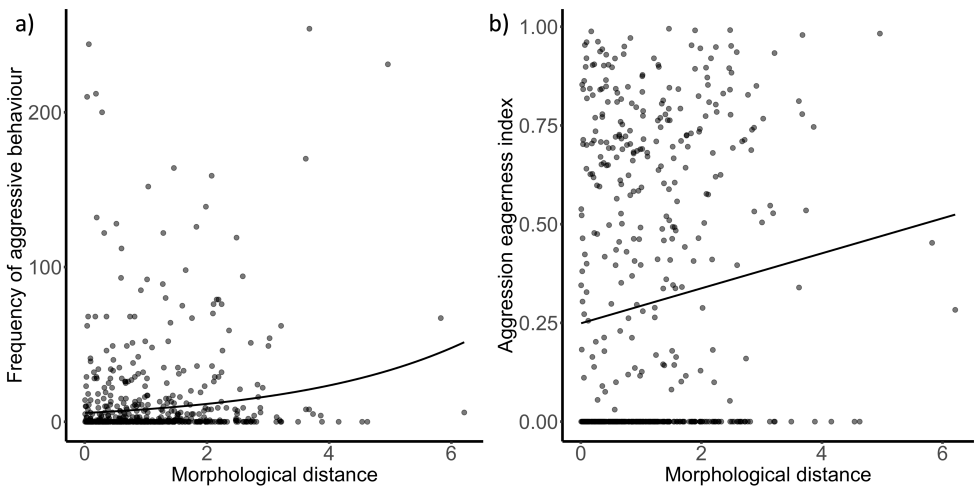


Figure 12. Effect of the distance between the male and female on the high-predation – low-predation morphology axis on a) level of female aggression b) female aggression eagerness index (1 indicates that female initiated aggression at the very start of the trial).

Mechanical isolation

In addition to inhibiting successful sperm transfer, incompatible genital morphologies can potentially lead to major genital injuries during interspecific mating events. However, this has rarely been investigated with the exception of some beetle species (Sota and Kubota 1998, Kubota *et al.* 2013). The genital morphology of both male and female Bahamas mosquitofish has predictably diverged between predation regimes (Heinen-Kay and Langerhans 2013, Anderson and Langerhans 2015): males and females adapted to the high-predation regime tend to have, respectively, more elongated genital tips and smaller genital openings compared to the low-predation regime. Thus, mechanical isolation would be expected to be higher in between-regime mating pairs. In mosquitofish, copulation typically happens in less than a second, with the male quickly thrusting the gonopodium into the gonopore of the female. Thus, incompatible genital structures between the female and the male could theoretically result in genital injuries. The males used in the mating trials for **paper II** were anesthetized right after the trials and their gonopodium tip was photographed under a microscope. The photographs revealed a large frequency of injuries in the bone structure of the gonopodium tips, ranging from smaller fractures to large missing pieces (Figure 13). We recorded the visible bone injuries and rated each individual on a binary scale of 0 (no visible injury) or 1 (visible bone injury). We predicted that the frequency of gonopodium injuries would be higher in males that had been paired with a female from the opposite predation regime. Gonopodium data was also reassessed from previous datasets to have a comparable number of males mated within their native population. The effect of different mating types (Figure 14) on male gonopodium injuries was evaluated with a GLMM (binomial distribution), which included female population and male population as random factors.

Supporting our hypothesis, the frequency of gonopodium injuries was the highest in males that had been paired with a female from a different predation regime ($\chi^2=31.39$, $Df = 3$, $p < 0.0001$, Figure 14). Furthermore, the direction of the mating seemed to have an effect on the likelihood of injuries, as the matings with a low-predation male and high-predation female had the highest frequency of injuries. This fits with the ecologically driven morphological differences, as the small genital openings of high-predation females would likely result in incompatibilities with the more rounded gonopodial tips of the low-predation males. The genital injuries were on a similar level in wild-caught fish, virgin laboratory-raised males, and males mated with their native predation regime, regardless of predation regime of origin. In males mated with a foreign female from a similar predation regime, the frequency of gonopodium injuries was on intermediate levels. The intricate bony structure of the gonopodium has likely evolved to maximise sperm transfer, and there is evidence that removal of these structures leads to less successful ejaculation, and thus likely inhibited sperm transfer in

future mating events (Chung *et al.* 2020). Large injuries, such as found in this study, should therefore act as a considerable fitness cost to the male in any future mating events, as they cannot regrow these structures. These results support the hypothesis that ecological divergence can drive mechanical isolation, which may result in significant fitness losses in males attempting to mate across predation regimes.

Additional trials where the male gonopodium was photographed before and after the mating trials confirmed that the majority of the injuries appear after the contact with a female. Still, since we did not record the behaviour of each mating pair for the whole time they were interacting, and even males raised separately from females had occasional injuries, we cannot confirm that these injuries result only from genital contact. Especially in the case of larger injuries, the injuries could have potentially been caused by female biting as a part of the aggressive behaviour, which was also higher in the same type of mating pairs. However, we could not detect any attempts to injure the gonopodium from the female in the recorded mating trials. It is therefore unlikely that the differences found in this study would be explained by other factors than ecology-driven incompatibilities in genital morphology.

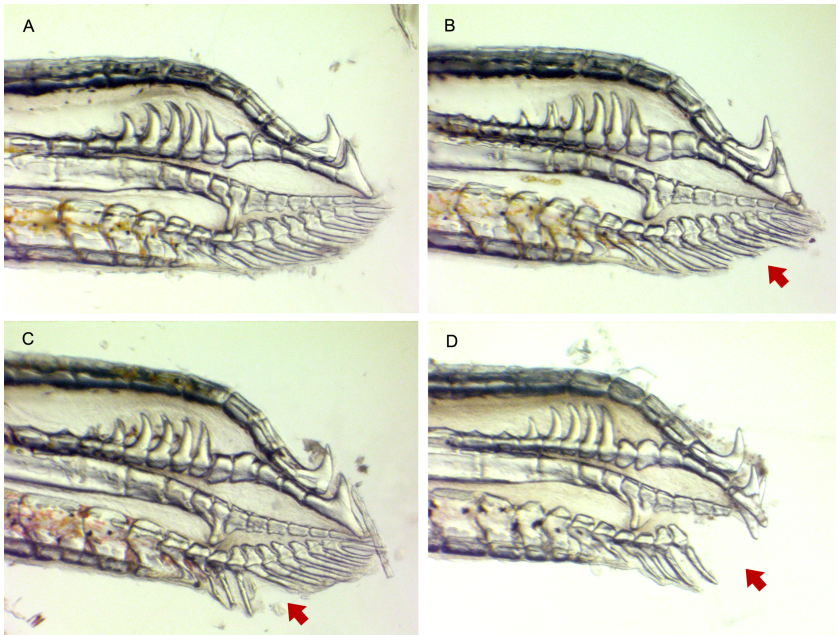


Figure 13. Photographs of the distant tip of the male gonopodium from a lateral view. (A) shows an example of an uninjured gonopodium, while (B)-(D) show examples of bone injuries.

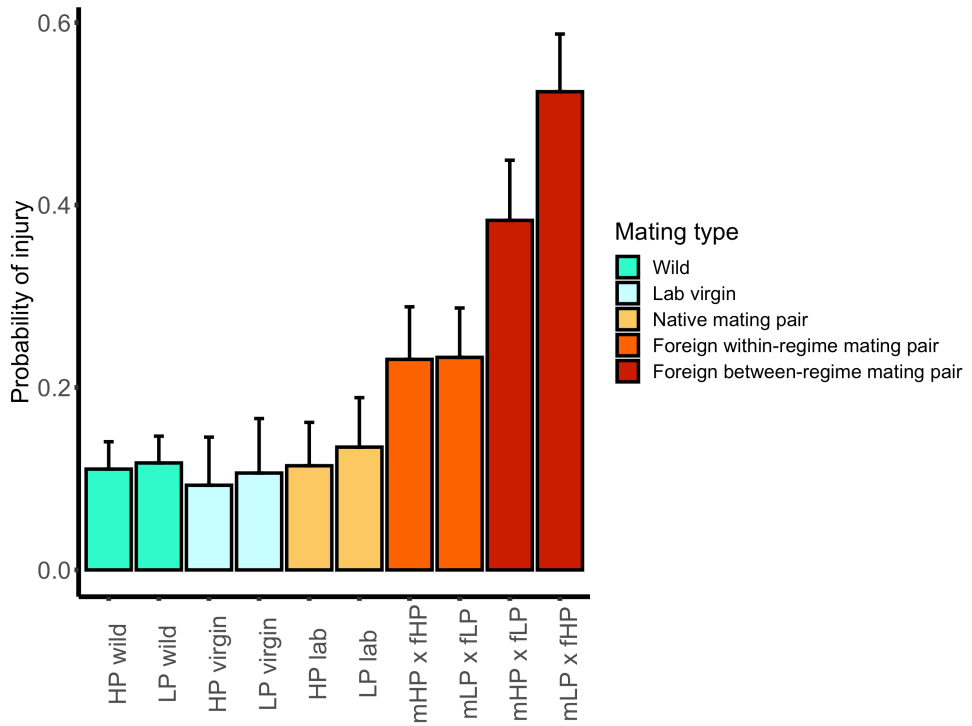


Figure 14. Probability of gonopodium injuries in males caught from the wild, raised in a lab without contact with females, or mated in the lab with either a native female, a foreign female from the same predation regime, or a foreign female from a different predation regime. HP = high-predation regime, LP = low-predation regime, m = male, f = female. Values represent estimated marginal means from a binomial GLMM, and the error bars denote standard error.

Hybrid morphology

Body shape in fishes is tightly connected to their swimming performance, and thus affects their ecology-driven traits such as predator avoidance and energy expenditure during swimming. Hybridization often leads to intermediate phenotypes, but it can also produce novel morphological traits that may either fall into a fitness valley or jump into a new fitness peak. We wanted to investigate whether morphology of hybrids produced between different mosquitofish populations was purely intermediate between parental phenotypes, or if they showed signs of novel body shape.

Using lateral photos of hybrids and pure-line fish, 16 landmarks representing body shape were recorded (Figure 11). All morphological analyses were done separately for females and males using the MorphoJ program. The landmark coordinates were transformed to remove the effects of position and size (Procrustes fit and a regression analysis to remove the allometric the size effects). We ran a CVA using the six different phenotypical combinations (HP pure-line, HP-HP hybrid, HP-LP hybrid, LP-HP hybrid, LP-LP hybrid, LP pure-line) as groups, with permutation tests as pairwise comparisons. In females, all pairwise groups significantly differed from each other, except for the LP-HP and LP-LP pair. In males, the non-significant pairwise groups were HP-HP – HP-LP, HP-HP – LP-HP, HP-LP – LP-HP and LP-HP – LP-LP. In females, the first two CV axes covered 82.9% of the overall morphological variance between groups, while in males they covered 90.1% of variance. In both males and females, the CV1 axis could be seen as a pure-line – hybrid phenotype axis (Figure 15). Likewise, CV2 seemed to be based on the ecological phenotype between high-predation and low-predation regime in both sexes. Interestingly, on the ecology-based CV2 axis, male hybrids produced between different predation regimes received intermediate scores, while the position of female hybrids on the axis was based on their maternal predation regime.

In summary, all hybrid groups were significantly different from the parental phenotypes in their overall body shape. In addition, most hybrid phenotypes could be categorized to distinct morphological groups based on their ecological origin, especially in females. The hybrids that most consistently differed from each other were the HP-HP and LP-LP hybrids. The ecology-based CV2-axis overall shows the same pattern as fast-start performance results from **paper III**, i.e. especially hybrids within the same predation regime resemble the pure-line phenotype, but in the hybrids produced between regimes, the direction of the cross may matter as well. The distinct hybrid body shape represented by the CV1 axis, however, may be reflected in novel behavioural traits as well. In addition, as the mate preferences of mosquitofish are partly based on body shape and the females seem to prefer body shapes similar to their own, the difference in morphology may also lead to sexual selection against hybrids.

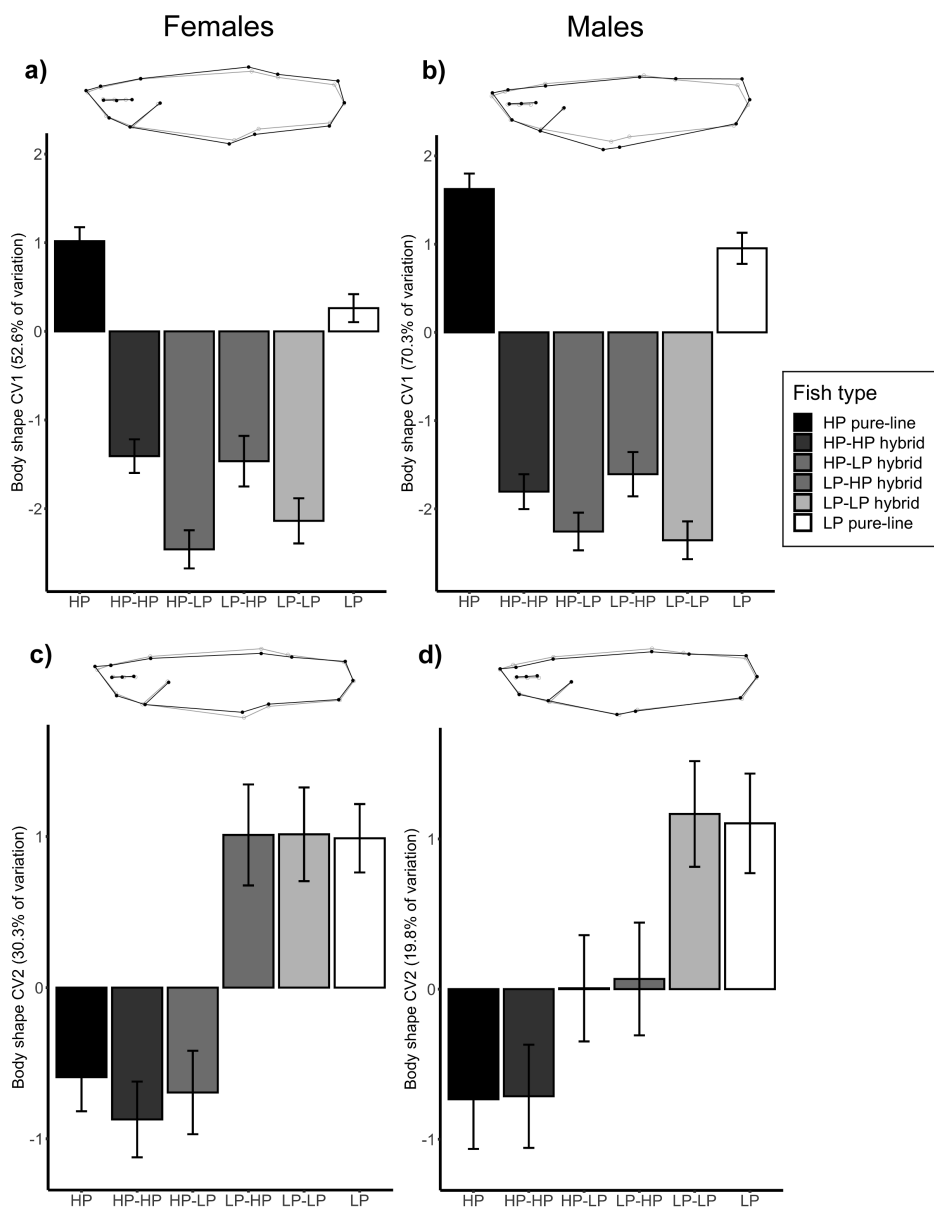


Figure 15. Morphological differences along the first two CV axes in female (a, c) and male (b, d) mosquitofish. The wireframe graphs represent morphological changes between the mean body shape (grey lines) and body shape towards the positive values of the CV axis (black lines). Values represent estimated marginal means from a GLMM, and the error bars denote standard error.

Conclusions

Predation has received fairly little attention as a driver of ecological speciation. In my thesis, I show that variation in predation risk can potentially restrict gene flow between populations through a number of different reproductive barriers. I show that predation can select against the maladapted phenotype of hybrids, but also that preceding isolating barriers can evolve as the by-product of divergent adaptations to different predation levels.

Instead of focusing on a just single isolating barrier, more and more recent studies set out to estimate the strength of the overall reproductive isolation as a sum of multiple barriers within a population/species complex (Matsubayashi and Katakura 2009, Baack *et al.* 2015, Lackey and Boughman 2017, Chin and Cristescu 2021). This has brought us closer to understanding how speciation operates, and allows us to compare how the role of each isolating barrier may change at different points of time after the lineages have diverged. However, most studies estimating the effect of multiple forms of reproductive isolation do not focus on a clear source of ecological divergence, with the exception of herbivores adapted to different host plants (Funk *et al.* 2002). My thesis brings us closer to estimating the full contribution of each major barrier in a system that has ecologically adapted to either the presence or absence of major predators. While additional studies are still needed to fully confirm the effects of some barriers, I am able to hypothesize the importance of each barrier in the event of increased gene flow. In addition to ecological speciation, this can help us to estimate which barriers can evolve already at an early stage of speciation.

If one were to name the current barriers to gene flow between the blue hole mosquitofish populations, the answer would technically be only one: geographical isolation. This situation might easily change in the future, however. The islands of the Bahamas are very flat, with most reaching only a few meters above the sea level at the highest point. At the same time, hurricane activity in the Caribbean is expected to increase as a result of global warming (Mousavi *et al.* 2011). It is not unlikely that a flood caused by a tropical storm could result in a sudden wave of immigrant mosquitofish into a foreign blue hole. What, then, would be the likelihood of these immigrants successfully mating and producing viable offspring with the native fish of this new population?

Based on the studies presented in this thesis, we know that the likelihood of gene flow between native fish and immigrants should depend on both the origin and sex of the immigrant. Starting with immigrant inviability, we knew that both female and male mosquitofish adapted to the low-predation regime are caught by predators more easily than fish from the high-predation regime (Langerhans 2009a). Based on **paper I**, it seems like female immigrants from high-predation regime might also struggle to compete against females in the low-predation regime. However, additional studies comparing the growth rates of fish interacting across predation regimes would be needed to fully confirm this. Males, on the other hand, may only suffer from immigrant inviability when a low-predation male arrives into a high-predation blue hole. Moving on to behavioural isolation, based on **paper II** we can assume that a female immigrant would have a higher chance at mating with the native fish than a male immigrant, as males do not show signs of strong discrimination towards foreign mates. Females seem to show stronger aggression towards foreign males, especially from the opposite predation regime, but how strongly the females can resist unwanted male mating attempts should still be confirmed in a natural setting. Based on the unpublished gonopodium injury data, mechanical isolation might be a major isolating barrier between populations, especially across different predation regimes. While possible injuries to the female genitalia remain to be investigated, it seems like mating with a female from a different predation regime may result in a major fitness cost for the male through a high risk of genital injuries. The hybridization success described in **paper III** confirms that regardless of the direction of the cross, mating pairs across different predation regimes should have a lower success rate of producing offspring, possibly as a sum of several prezygotic different barriers. Finally, based on **paper III**, the hybrid offspring produced across predation regimes seem to suffer from higher mortality due to intrinsic incompatibilities, but this is only prominent in female hybrids. In addition, hybrids produced between predation regimes may face stronger ecological selection in the high-predation habitat compared to the low-predation regime.

Taken together, it seems clear that almost any isolating barrier is stronger between different predation regimes, in comparison to populations within the same regime. It is likely that the strongest reproductive isolation would appear between a male adapted to the low-predation regime migrating to the high-predation regime. In contrast, a female migrating to a blue hole with a similar predation regime might have a mating success almost on the same level as native females. Premating isolation is often expected to play a larger role in the early stage of speciation, and it has also been the most studied in terms of ecological speciation. While I also found support that premating isolation should restrict the gene flow between predation regimes, some other reproductive barriers may be at least equally important in this system. Namely, mechanical isolation and intrinsic hybrid inviability have rarely been studied in the context of ecological selection, so finding support of their importance widens the scope of how ecological speciation can proceed. While in **paper III** I tested the performance of hybrids in simple

ecological traits, the results from **paper IV** show that studying hybrid behaviour in the natural environment can reveal new mechanisms that significantly contribute to hybrid fitness. Furthermore, mosquitofish hybrids could be selected against by sexual selection, based on their different body shape compared to pure-line fish, but this remains to be further investigated. Although none of the reproductive barriers in the mosquitofish system seem complete, together they could already now significantly reduce gene flow between different predation regimes, leading to ecological speciation.

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