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**Ekologie chování gekončíka nočního (*Eublepharis macularius*)
Behaviour and ecology of leopard geckos (*Eublepharis macularius*)**

Dizertační práce

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Prohlašuji, že jsem dizertační práci zpracovala samostatně a uvedla jsem všechny použité informační zdroje a literaturu. Dále prohlašuji, že jsem se v uvedených rukopisech podílela na všech fázích jejich přípravy a můj celkový podíl na rukopisech odpovídá pořadí a počtu spoluautorů. Zároveň prohlašuji, že jsem nepředložila práci ani její podstatnou část k získání jiného nebo stejného akademického titulu.

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Poděkování

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Abstrakt

V průběhu evoluce přírodní výběr selektuje jedince, kteří volí tu nejlepší strategii pro přežití a největší reprodukční úspěch. Z evolučního hlediska hraje hybridizace významnou roli v procesu speciace. Zvolení vhodné antipredační strategie vzrůstá šance jedince na přežití. Ve své dizertační práci jsem se zabývala těmito dvěma tématy a jejich vlivu na fitness u gekončíka nočního (*Eublepharis macularius*, Eublepharidae). Oproti jiným skupinám obratlovců je záznamů o hybridizaci a jejím vlivu na fitness (fertilitu, přežívání) hybridů u ještěřů málo, ačkoliv hybridizují i geneticky vzdálené druhy. Efekt hybridizace na fitness byl studován u dvou druhů gekončíků (*E. macularius* a *E. angramainyu*). Zda jsou ochotni se křížit s cizím druhem a jaký to má dopad na fitness hybridů. Podobně byli studovány u geneticky příbuznějších forem komplexu druhů kolem *E. macularius* případné prekopulační omezení a výhody a ztráty spojené s hybridizací. Analýza dat ukázala, že ještěři hybridizují i mezi velmi fylogeneticky vzdálenými druhy a dokonce jsou „úspěšnější“ než ptáci nebo želvy. Překvapivě se v naší studii neprokázal vztah mezi genetickou vzdáleností rodičovských druhů a sterilitou anebo neživotaschopností hybridů. Alespoň částečná fertilita F1 hybridů a tok genů se vyskytoval i u hybridizací mezi geneticky nejvzdálenějšími rodičovskými druhy. Zjistili jsme, že nejsou vytvořeny dostatečné prekopulační zábrany, které by bránily jak mezidruhové, tak vnitrodruhové hybridizaci. Významné bylo zjištění, že mezidruhové křížení druhů *E. angramainyu* a *E. macularius* je prvním záznamem o křížení u druhů s teplotně určeným pohlavím (TSD) u ještěřů. Zároveň patří k hybridizaci mezi geneticky nejvzdálenějšími druhy ještěřů ve srovnání s dostupnou literaturou. Na základě morfologických analýz a analýzy zbarvení se liší jak rodičovské druhy *E. angramainyu* a *E. macularius*, ale také jejich F1 hybridů. Druh *E. angramainyu* roste pomaleji a větší velikosti dosáhne díky delšímu období exponenciálního růstu. F1 hybridů jsou životaschopní a fertilní a introgrese je umožněna díky zpětnému křížení. Mezidruhová hybridizace, s výjimkou F2 hybridů, nemá žádná poškození a ani horší životaschopnost nebo růst. Na základě toho lze očekávat, že ještěři hybridizují častěji, a i mezi geneticky rozdílnějšími druhy, než se dodnes ví. Podařilo se nám zdokumentovat změny antipredační strategie v průběhu ontogeneze u druhu *E. macularius*. Zatímco mláďata odrazují predátora vokalizací, dospělci utíkají a mají výhodu z kryptického zbarvení.

Abstract

During evolution, the natural selection favours individuals with the best survival strategy and the highest reproductive success. From the evolutionary point of view, hybridization plays an important role in the process of speciation. Avoiding predators by choosing the most appropriate antipredator strategy increases the animal's chances of survival as well. Studying fitness consequences of hybridization and predation in *Eublepharis macularius* (Eublepharidae) was the main objective of this theses. Compared to other vertebrate groups, the reliable records on hybridization and its effect on the hybrid's fitness (fertility, survival) in lizards are scarce, despite their ability to hybridize between genetically distant species. These effects were examined in two species of eyelid geckos (*E. macularius* and *E. angramainyu*). We aimed to discover whether they were willing to hybridize with a heterospecific species and how the fitness of the hybrids would be affected. Similarly, were studied more genetically related forms of *E. macularius* species complex, the potential precopulatory barriers, and fitness cost of this hybridization. Analysis of published data has shown that the lizards hybridize between very phylogenetically distant species and are even "more successful" than birds or turtles. Surprisingly, we didn't prove relationship between the genetic distances of parental species and the sterility or unviability of hybrids. The F1 hybrids were typically at least partially fertile and the genetic introgression was possible. The sufficient precopulatory barriers to prevent both the interspecies and the intraspecies hybridizations were not found. Significant was the finding that the interspecific hybridization between the *E. angramainyu* and *E. macularius* was the first record of crossing the species with temperature-dependent sex determination (TSD) in lizards. At the same time, this cross belongs to the hybridization between genetically most distant species in lizard compared with the available literature. Analyses of morphometric and colour traits confirmed the phenotypic distinctiveness of both parental species of *E. angramainyu* and *E. macularius*, as well as their F1 hybrids. *E. angramainyu* species grew more slowly and the larger size was attained by a longer period of exponential growth. I demonstrated that F1 hybrids were viable and fertile and the introgression might be enabled via backcrossing. The interspecific hybrids, except for F2 generation, displayed neither malformations nor reduced survival or growth. Based on these findings, the lizards can be expected to hybridize more frequently and even between more genetically distinct species than what has been known. Finally, optimal antipredator strategy changes over ontogeny were documented in *E. macularius*, as juveniles deter a predator by vocalization, while the adults rather escape and benefit from their cryptic colouration.

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I.

Jančúchová-Lásková, J., Landová, E. & Frynta, D. (2015) Are genetically distinct lizard species able to hybridize? A review. *Current Zoology* 61: 155-180.

II. Landová, E., **Jančúchová-Lásková, J.,** Kratochvíl, L., Polák, J. & Frynta, D. Divergence in sexual behaviour during distant and close hybridization in eublepharid geckos: experimental crossing of *Eublepharis macularius* and its congeners (*manuscript*).

III. **Jančúchová-Lásková, J.,** Landová, E. & Frynta, D. (2015) Experimental crossing of two distinct species of leopard geckos, *Eublepharis angramainyu* and *E. macularius*: viability, fertility and phenotypic variation of the hybrids. *Plos One* 10.

IV. Frynta, D., **Jančúchová-Lásková, J.,** Frýdlová, P. & Landová, E. Fast or slow? A comparative study of body weight trajectories in three species of the genus *Eublepharis* and their hybrids (*manuscript*).

V. Landová, E., **Jančúchová-Lásková, J.,** Musilová, V., Kadochová, S. & Frynta, D. (2013) Ontogenetic switch between alternative antipredatory strategies in the leopard gecko (*Eublepharis macularius*): defensive threat versus escape. *Behavioral Ecology and Sociobiology* 67: 1113-1122.

1. Úvod

Gekončík noční (*Eublepharis macularius*) se v současné době těší oblibě nejen zájmových chovatelů díky svému atraktivnímu vzhledu, ale i vědců. Již od 90. let 21. století se začal využívat ke zkoumání chemorecepce (Mason & Gutzke 1990), vlivu inkubační teploty na určení pohlaví u mláďat (Viets et al. 1993), ale i na chování jedince v dospělosti (Flores et al. 1994; Tousignant & Crews 1995; Rhen & Crews 1999). V americké laboratoři kolem profesora Crewse vzniklo mnoho neurobiologických prací zkoumající vliv hormonů na struktury mozku a chování gekončíka nočního v kontextu právě inkubační teploty (Flores & Crews 1995; Crews et al. 1996; Coomber et al. 1997; Crews et al. 1997). Později se vědci zaměřili na jeho rozpoznávací schopnosti v kontextu sociálního chování (Cooper & Steele 1997; Steele & Cooper 1997; LaDage & Ferkin 2006; LaDage & Ferkin 2007). A neméně zajímavé práce vznikaly a vznikají na témata ekomorfologická či fyziologická na Katedře zoologie u zakladatele chovu a výzkumu gekončíků doc. Daniela Frynty a Mgr. Zuzany Starostové, PhD. a na Katedře ekologie pod záštitou prof. Lukáše Kratochvíla, PhD. (Kratochvíl & Frynta 2002; Kratochvíl & Frynta 2003; Kratochvíl & Frynta 2006; Starostová et al. 2013a; Starostová et al. 2013b; Schořálková et al. 2017; Starostová et al. 2017). Ukázalo se, že je to vhodný modelový organizmus ještěra, který není náročný na chov, nemá příliš speciální dietetické požadavky a při dobré péči se velmi dobře množí a dožívá se vysokého věku (až 25 let, Frynta osobní sdělení).

V rozporu s poměrně velkou prozkoumaností tohoto druhu po stránce fyziologické, je poměrně mizivé procento prací pojednávající o ekologii a etologii tohoto druhu z přirozeného prostředí. Vysvětlení je celkem prozaické. Tento druh pochází z politicky velmi nestabilního prostředí Pákistánu, východního Afghánistánu a severozápadní Indie. Jen pár prací se zmiňuje o přirozeném prostředí gekončíka nočního a o jeho ekologii a chování v přírodě (Minton 1966; Khan 1999). Lépe je prozkoumaná ekologie např. u druhu *Goniurosaurus kuroiwae* (Tanaka & Nishihira 1987; Tanaka & Nishihira 1989) nebo *Coleonyx variegatus* (Parker 1972) ze stejné čeledi Eublepharidae. Přiznám se, že jsem neměla tak vysoké cíle, abych přispěla k výzkumu tohoto gekončíka v jeho přirozeném prostředí. I když při několika hovorech s docentem Fryntou padaly různé „troufalé“ nápady na výzkum v jejich domovině, nakonec zůstalo „pouze“ u několikaletého sledování mnoha stovek gekonů v chovu datující se už od dob mého

bakalářského studia. Zaměřila jsem se především na jejich morfologii, reprodukci, růst, přežívání a antipredační chování. Další velké téma, kterým jsem se zabývala, bylo, zda hybridizace hraje významnou roli v evoluci vzniku druhů u ještěřů. Snažila jsem se z dostupné literatury dopátrat, kam až lze zajít při křížení různě geneticky odlišných druhů a jaký to má následně dopad na fitness u případných hybridů. Ty samé otázky jsem si pak kladla při vlastních experimentech se dvěma druhy gekončků, *E. angramainyu* a *E. macularius* v rámci diplomové práce. Jelikož mě zajímal vliv hybridizace na fitness a fertilitu hybridů nejen první generace (F1), ale i druhé (F2) a hybridů ze zpětného křížení (B) a s ohledem na generační dobu gekončků, přerostla tato studie do mé dizertační práce, kdy tyto hybridy vyššího řádu konečně dosáhli dospělosti. Výsledkem tohoto bádání je dizertace sestávající ze třech publikovaných článků a dvou rukopisů odeslaných do časopisů. Díky možnosti zkoumání velkého množství jedinců, tak mohl vzniknout unikátní soubor na sebe navazujících informací o vlivu hybridizace na epigamní chování, investici matek do reprodukce, líhnivost vajec, sledování růstu mláďat až do dospělosti a následné ověření fertility těchto jedinců. Mimo to bylo získáno velké množství dat stejného charakteru o rodičovských druzích. O vzácnějším druhu *E. angramainyu* podobné údaje zcela chybí. Velmi zajímavým objevem pak bylo popsání změny v antipredačních strategiích u druhu *E. macularius* během ontogeneze. Pro celou studii bylo zcela zásadní, že jsem mohla pracovat se zvířaty pocházejícími z přírody a jejich potomky z první generace. Tím jsme mohli z výsledků našich experimentů vyloučit vliv inbreedingu na morfologii, líhnivost nebo reaktivitu gekončků.

2. Cíle práce

Obecnými cíli práce bylo zjistit, jak dané chování v kontextu hybridizace či predace teoreticky přispívá k vyššímu fitness jedince. Tyto otázky jsem řešila v širším úhlu behaviorální ekologie neboli ekologie chování. Ta studuje, jak dané životní strategie živočichů mohou přispívat k jejich přežití (Davies et al. 2012). Nejčastěji se ptáme jak a proč se konkrétní živočich chová a hledáme možné ultimátní příčiny, které by takové chování osvětlily. V této práci se ptám převážně nejen na příčiny, ale i na důsledky daného chování a interpretuji je ve smyslu evoluční biologie. Pouze poslední práce kombinuje pohled behaviorálně ekologický a ukazuje, jak se výhody a nevýhody antipredačního chování mohou měnit v průběhu ontogeneze.

Hlavním cílem dizertační práce bylo najít odpovědi na následující otázky, jež jsou uvedeny spolu s hlavními úkoly a se třemi publikacemi a dvěma rukopisy, kde jsou výsledky podrobně diskutovány:

- I. Jak moc geneticky vzdálené druhy ještěřů se mohou ještě křížit a jaký má dopad hybridizace na jejich fitness – fertilitu a životaschopnost?

Jančúchová-Lásková, J., Landová, E. & Frynta, D. 2015: Are genetically distinct lizard species able to hybridize? A review. *Current Zoology* 61, 155-180.

- II. Existuje mezi geneticky vzdálenějšími druhy *E. angramainyu* a *E. macularius*, popřípadě mezi formami komplexu druhu *E. macularius* prekopulační bariéra bránící vzájemné hybridizaci?

Provést hybridizační experimenty mezi blízkými příbuznými formami gekončků druhu *E. macularius* a dále mezi geneticky vzdálenějšími druhy *E. angramainyu* a *E. macularius* a zjistit a popsat případné rozdíly v epigamním chování a preferencích sexuálního partnera.

Landová, E., Jančúchová-Lásková, J., Kratochvíl, L., Polák, J. & Frynta, D. Divergence in sexual behaviour during distant and close hybridization in eublepharid geckos: experimental crossing of *Eublepharis macularius* and its congeners (*manuscript*).

- III.** Jaký má dopad hybridizace mezi druhy *E. angramainyu* a *E. macularius* na fitness hybridů v různých generacích? Existují mezi těmito druhy postzygotické reprodukčně izolační bariéry?

Po úspěšné hybridizaci, sledovat u hybridů první (F1) a druhé generace (F2) a hybridů ze zpětného křížení s oběma rodičovskými druhy (B_M) a (B_A) různé koreláty fitness – hmotnost vajíček, hmotnost mláďat při líhnutí, růst, přežívání a fertilitu po dosažení dospělosti. Tyto údaje pak porovnat s rodičovskými druhy.

Jančúchová-Lásková, J., Landová, E. & Frynta, D. 2015: Experimental crossing of two distinct species of leopard geckos, *Eublepharis angramainyu* and *E. macularius*: viability, fertility and phenotypic variation of the hybrids. Plos One 10.

- IV.** Je velikost dospělého gekončíka určena spíše rychlostí růstu nebo časem exponenciálního růstu? Ovlivní hybridizace negativně růstové parametry?

Sledovat druhy *E. angramainyu* a *E. macularius* a jejich morfologicky odlišné populace od vylíhnutí po dospělost. Porovnat růstové parametry mezi rodičovskými druhy/formami a jejich hybridy.

Frynta, D., Jančúchová-Lásková, J., Frýdlová, P. & Landová, E. Fast or slow? A comparative study of body weight trajectories in three species of the genus *Eublepharis* and their hybrids (manuscript).

- V.** Mění se antipredační strategie gekončíka nočního během jeho ontogeneze?

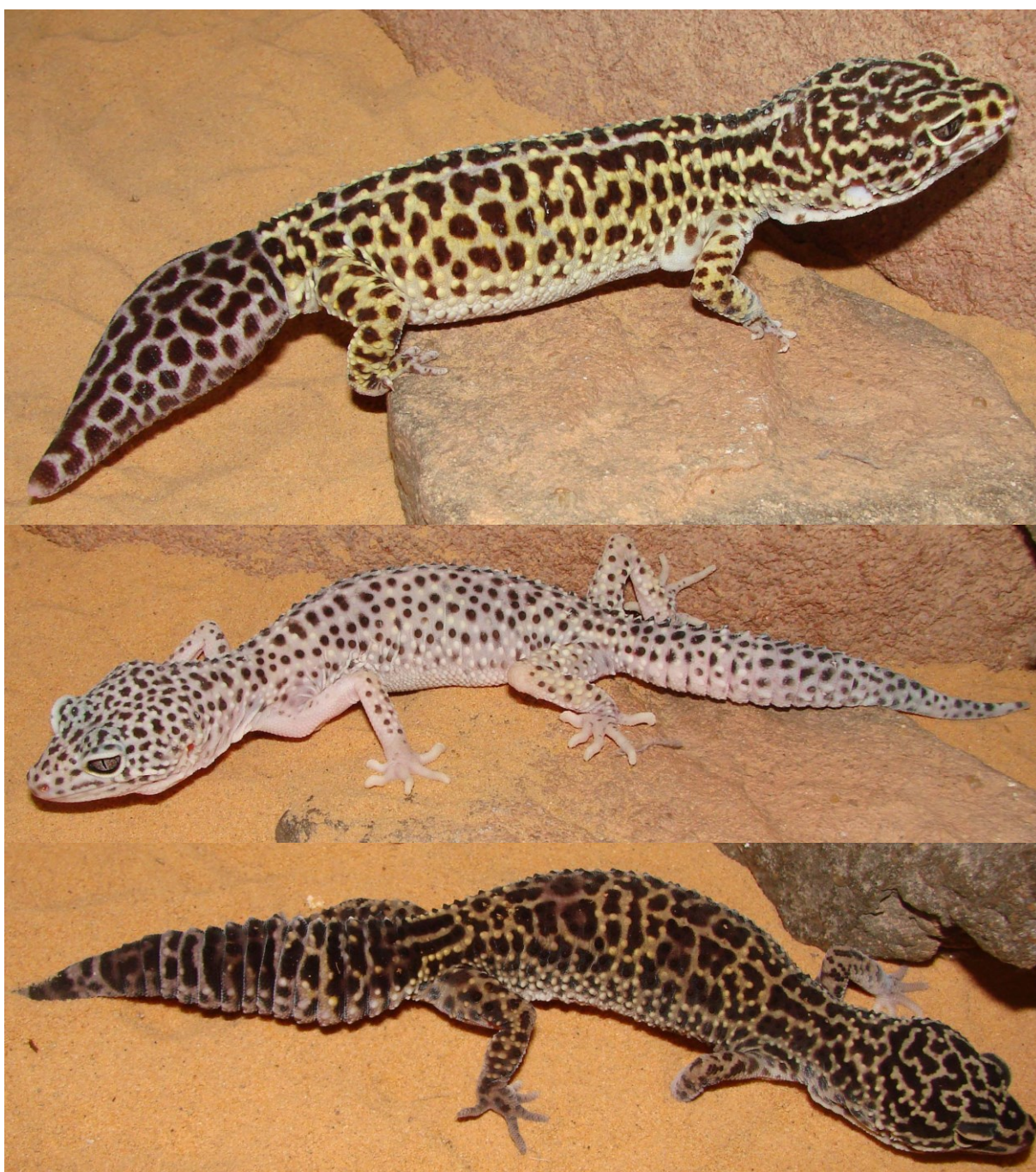
Sledovat antipredační chování na jednoduchý taktilní stimul během ontogeneze – od narození po dospělost.

Landová, E., Jančúchová-Lásková, J., Musilová, V., Kadochová, S. & Frynta, D. 2013: Ontogenetic switch between alternative antipredatory strategies in the leopard gecko (*Eublepharis macularius*): defensive threat versus escape. Behavioral Ecology and Sociobiology 67, 1113-1122.

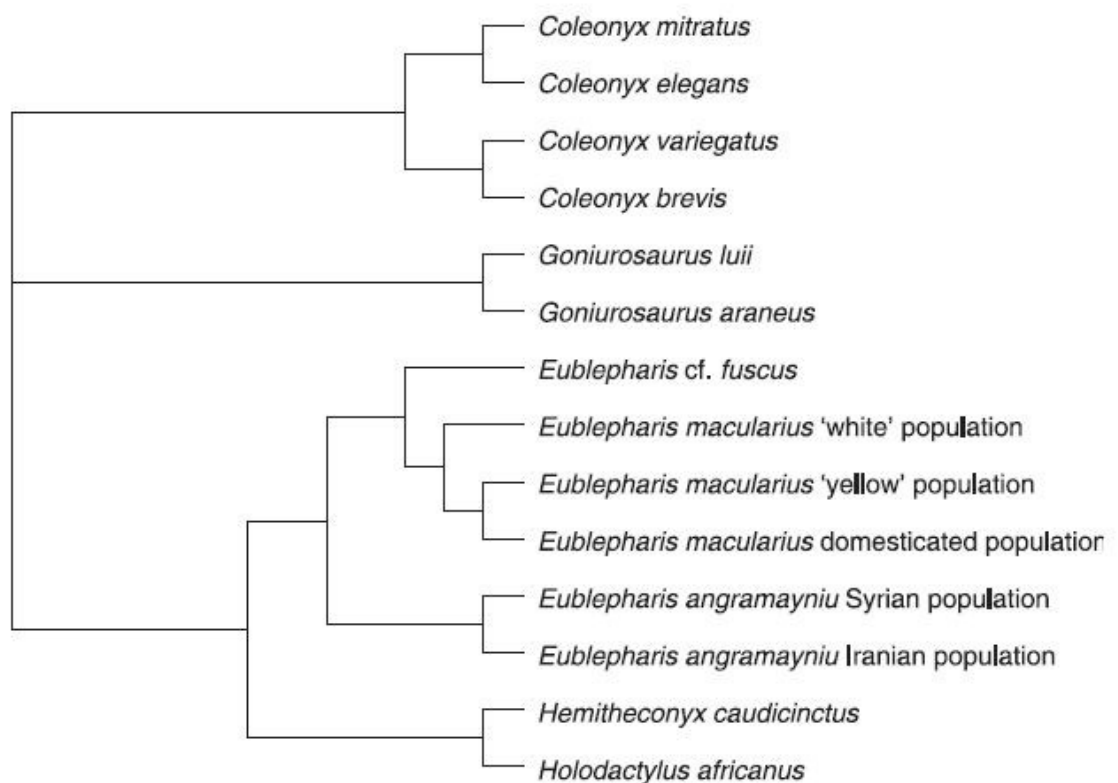
3. Výsledky prací v širším kontextu

3.1. Gekončiči *E. macularius* a *E. angramainyu* (čeleď Eublepharidae)

Čeleď Eublepharidae je malá monofyletická skupina, sesterská k ostatním gekonům (Han et al. 2004), do které patří šest rodů, včetně rodu *Eublepharis* Gray, 1827. Tato čeleď je poměrně velmi variabilní ve velikosti a je velikostně dimorfní mezi pohlavími. Platí zde Renschovo pravidlo, kdy u větších druhů je větším pohlavím samec a u menších druhů je větším pohlavím samice (Kratochvíl & Frynta 2002). Největším zástupcem této čeledi je druh *E. angramainyu* Anderson et Leviton, 1966. Největší samec v našem chovu dorostl velikosti 165,6 mm (SVL) při hmotnosti 234 g a největší samice 152,5 mm a 173,7 g. K nejmenším zástupcům patří *Coleonyx brevis* Stejneger, 1893 s průměrnou velikostí 61 mm a hmotností 4,6 g (Starostová et al. 2005). V evoluci této čeledi nejspíše došlo jak ke zvětšování, tak ke zmenšování velikosti těla (Grismer 1988; Starostová et al. 2005). Mezi vědci i chovateli si získal oblibu středně veliký druh *E. macularius* Blyth, 1854, který pochází z Pákistánu, východního Afghánistánu a severozápadní Indie. Jen pár prací se zmiňuje o přirozeném prostředí gekončička nočního a o jeho ekologii a chování v přírodě (Minton 1966; Khan 1999). Někteří poukazují na to, že v rámci tohoto druhu existují více forem či poddruhů, které se liší nejen morfologicky, ale i geneticky (Seufer et al. 2005; Starostová et al. 2005). Při mé práci jsem pracovala s třemi formami, které se lišily nejen barevně, ale i velikostně a tvarem těla (Obr. 1). Forma, která má charakteristické zbarvení druhu *E. macularius*, jsme nazvali „žlutá“ a odpovídá označení „yellow population“ v publikaci Starostová et al. 2005. Geneticky velmi podobná, ale morfologií odlišná forma „bílá“ odpovídá označení „white population“. Námi označovaná „tmavá“ forma odpovídá *E. cf. fuscus* Börner, 1981 v publikaci Starostová et al. (2005) viz. Obr. 2.



Obr. 1 Žlutá, bílá a tmavá forma gekončků druhu *E. macularius* a *E. sp.*



Obr. 2 Fylogeneze čeledi Eublepharidae (Kratochvíl & Frynta 2002; Starostová et al. 2005).

Taxonomie tohoto druhu i celého rodu není příliš jasná (Das 1997; Seufer et al. 2005; Mirza et al. 2014), a proto prozatím používáme označení „tmavá“ forma. Čím se však krom tmavého zbarvení odlišuje od „žluté“ či „bílé“ formy, je výrazně menší velikost těla. Průměrná délka SVL pro samce „tmavé“ formy v našem chovu byla 104,6 mm a hmotnost 25 g (n = 9) a pro samice 104,5 mm a 32,2 g (n = 14). Pro „žlutou“ formu to bylo 136,8 mm a 62,2 g u samců (n = 12) a 127,2 mm a 52,7 g pro samice (n = 55). A pro samce „bílé“ formy byly průměrné hodnoty SVL a hmotnosti 131,5 mm 53,1 g (n = 11) a pro samice 126,1 mm 48,3 g (n = 27). Všechna zvířata v době měření a vážení byla starší než dva roky, tj. sexuálně dospělá a plně vzrostlá. Ačkoliv mají tato zvířata neukončený růst, v této době už jsou přírůstky minimální (**Publikace IV.**; nebo podobně u varanů, Frynta et al. 2010). Rozdíl ve velikosti je markantní hned po vylíhnutí jak mezi druhy *E. angramayniu* a *E. macularius* (**Publikace III.**), tak pro „žlutou“ a „tmavou“ formu.

Průměrná hmotnost „žluté“ formy ($n = 27$) je 3,8 g; „tmavé“ formy ($n = 30$) 2,4 g; „bílé“ formy ($n = 30$) 3,9 g a druhu *E. angramainyu* ($n = 6$) 8,9 g. Tyto výsledky by odpovídaly maternálnímu efektu na velikost mlád'at, resp. vajec (Kratochvíl & Frynta 2006). Oba druhy gekončků mají ve většině případů invariantní snůšku dvou vajec až osm krát v jejich reprodukční sezóně od března do poloviny srpna (Werner 1972; Seufer et al. 2005; Kratochvíl & Frynta 2006). Investice matky do jedné snůšky je poměrně velká (14–16 % hmotnosti). Dle Kratochvíl & Frynta (2006), matky maximalizují investici do vajec a jejich velikost je omezena velikostí samice, resp. velikostí její břišní dutiny. Tento izometrický vztah velikosti matky a vajíčka je poměrně ojedinělý. Běžněji je dokumentována alometrie, kdy velikost vajíčka se zvětšuje pomaleji, než by odpovídalo velikosti těla. Větší druh má tak relativně menší vajíčko než malý druh (Kratochvíl & Frynta 2006).

Druhy *E. macularius* a *E. angramainyu* se mezi sebou neliší jen ve velikosti mlád'at a dospělců, ale i ve tvaru těla, charakteristického vzoru skvrn na hlavě (**Publikace III.**) a struktuře subdigitálních lamel prstů. Druh *E. angramainyu* má subdigitální lamely hladké, druhu *E. macularius* hrbokaté (Mirza et al. 2014). V diskriminační analýze na základě 14 rozměrů na těle a hlavě se ukázalo, že druh *E. angramainyu* má relativně delší nohy než *E. macularius* a dále je pro něj charakteristický menší počet podlouhlých tmavých skvrn na hlavě namísto většího počtu menších okrouhlejších skvrnek typických pro druh *E. macularius* (**Publikace III.**). Ukázalo se, že větší druh *E. angramainyu* doroste dospělé velikosti za delší dobu a roste pomaleji oproti menšímu druhu a formám (**Publikace IV.**).

E. angramainyu je méně běžný druh, který obývá oblast bývalé Mezopotámie a jihozápadního Íránu (Anderson 1999). Výskyt obou druhů odděluje Íránské Plato a pohoří Zagros (Göçmen et al. 2002; Seufer et al. 2005). Jak geologické, tak genetické poznatky naznačují, že tyto dva druhy se oddělily před více jak 12–15 miliony lety (detaily v **Publikaci III.**). Informace o ekologii a chování druhu *E. angramainyu* jsou sporadické. Oba druhy se vyskytují ve stepních porostech s kamenitou půdou od nížin až po pohoří Zagros a Himaláje. Z dostupných záznamů se zdá, že *E. macularius* je rozšířenější druh s širší ekologickou nikou, který se vyskytuje v oblastech s velkými denními i ročními výkyvy teplot -14 až 48 °C a který obývá variabilnější biotopy i pozměněné člověkem. V jižní části areálu obývá i subtropický suchý les s větší mírou dešťových srážek, než je tomu na severu rozšíření (Khan 1999; Göçmen et al. 2002; Seufer et al. 2005; Smid et al. 2014;

Safaei-Mahroo et al. 2015). Tento závěr však může být dán právě nedostatkem informací o vzácnějším druhu *E. angramainyu*.

U obou druhů je větším pohlavím samec. Ti jsou teritoriální a značně agresivní k jiným samcům. K vyznačování teritoria používají sekret z preanálních pórů (Brandstaetter 1992) a mají oproti samicím relativně větší hlavu, což je může zvýhodňovat při samčích agresivních interakcích (Kratochvíl & Frynta 2002). Samice nejsou agresivní, ale během interakcí se samcem, pokud samec nereflktuje její případné odmítnutí, které signalizuje zdviženým a horizontálně se vlnícím ocasem, může samice na samce zaútočit. Útoky jsou však krátké a spíše výhružné. Samice většího druhu, *E. angramainyu*, používá kousnutí k odmítnutí samce častěji než samice *E. macularius* (**Publikace II.**). Jak vyplývá z našich experimentů o epigamním chování gekončků (**Publikace II.**), oba druhy se jinak ve vzoru epigamního chování neliší. Hlavními prvky epigamního chování samce je dvoření samce vibrací ocasu ve vysoké frekvenci. To trvá jen několik málo sekund a samec jej během dvoření několikrát opakuje. Tuto vibraci ocasem používá i samice, ale jen v kontextu potravního chování. Obě pohlaví mohou těsně před útokem na hmyz, kterým se živí, krátce vibrovat ocasem (Brandstaetter 1992). Druhý významný prvek dvořícího chování je jemné okusování samice, kdy samec drží samici v čelistech jen za malý kousek kůže. Samec se nejčastěji zakusuje nejprve v oblasti ocasu a pak postupuje přes záda na oblast za krkem. Některé části může přeskočit, a dokonce může rovnou uchopit samici za krkem. To je iniciální pozice těsně před samotnou kopulací. Samec částečně vyleze na samici, obejmě ji a ocasem se snaží dostat pod ten její, aby získal přístup ke kloace. Pokud je samice receptivní, nadzdvihne ocas a umožní samci kopulaci. Ta trvá v průměru 26 sekund. Ty nejdelší kopulace trvaly téměř minutu. Pokud samice není receptivní a pářit se nechce, volí mezi útekem a útokem, přičemž útek naprosto převládá. Stejně jako samec signalizuje vibrací ocasu záměr se pářit, samice signalizuje odmítnutí příkrčením, ztuhnutím a popřípadě vlnivým pohybem ocasu. Pokud je ochotná se pářit, tak vyčkává v klidu a toleruje samčí okusování. To, zda dojde ke kopulaci, záleží na receptivitě samice. Případ znásilnění, kdy samice se zjevně bránila, a přesto ji samec kousáním donutil k páření jsme za celou dobu našich experimentů zaznamenali jen jednou. Nucená kopulace se objevuje častěji u příbuzných druhů *Coleonyx elegans* a *C. mitratus* (Eublepharidae), kde došlo k výraznému zkrácení dvořící fáze samce (Zelená, Kratochvíl & Frynta nepublikovaná data).

Ukázalo se, že reprodukční, ale i agresivní chování u druhu *E. macularius* je ovlivněno sociální zkušeností a teplotou, v níž se zvířete, resp. vajíčka inkubovala (Gutzke & Crews 1988; Flores et al. 1994; Sakata et al. 2002). Tito gekončíci totiž patří do skupiny zvířat, jejichž pohlaví je určeno teplotou, v které se inkubují vajíčka, mají tzv. temperature sex determination (TSD), oproti klasickému geneticky určenému pohlaví (GSD) (Wagner 1980; Bull 1987; Viets et al. 1994). Ukázalo se, že určení pohlaví souvisí i s přítomností či absencí pohlavních chromozomů. Druhy s teplotně určeným pohlavím tak sdílí stejný genotyp jak samci, tak samice a nemají rozlišené pohlavní chromozomy (Valenzuela et al. 2003; Pokorná & Kratochvíl 2009; Pokorná et al. 2010). Vejce tohoto druhu se můžou inkubovat při teplotě v rozmezí 26-34 °C. Při teplotě 31-33 °C se líhne větší procento samců a při nižší nebo vyšší teplotě se naopak líhne větší procento samic (Bull 1987; Gutzke & Crews 1988; Viets et al. 1993). Nicméně se ukázalo, že samice se při snůšce vyhýbají pro ně extrémním teplotám (na okrajích teplotního rozmezí) a preferují teplotu 28-28,9 °C (Bull et al. 1988; Bragg et al. 2000).

U druhu *E. angramainyu* se zatím teplotně určené pohlaví neprokázalo. Důvodem jsou jeho nízké počty v chovech a nedostatek informací o jeho reprodukci. Na základě jeho fylogenetické blízkosti s druhem *E. macularius* a stejně tak shodného genotypu mezi pohlavím (Pokorná et al. 2010) se dá předpokládat, že by i u něj mohlo být pohlaví dáno inkubační teplotou. Například z výsledků našich experimentů můžeme říct, že jeho teplotní optimum pro inkubaci vajec bude oproti *E. macularius* vychýlené k nižším hodnotám. Inkubační teplota 28 °C byla pro mláďata *E. angramainyu* letální (Kratochvíl, osobní sdělení). Při 26 °C se líhla mláďata jen ve 34 % oproti vysoké líhivosti 92 % u druhu *E. macularius* (při jeho optimální inkubační teplotě 28 °C) (**Publikace III.**). Samozřejmě za nižší líhivosti mohou stát i jiné faktory, které bohužel zatím neznáme.

Jak jsem zmínila výše, je prokázané, že inkubační teplota ovlivňuje i chování u druhu *E. macularius*. Například samci, kteří se inkubovali za vyšší teploty, kdy se líhnou více samci, jsou agresivnější než samci z teploty, kdy se líhnou více samice. Samici z nižších teplot jsou také více sexuálně aktivní. Zvýšená agresivita se objevuje i u samic z vyšších teplot. Tyto samice jsou pak často i odmítavé k samčí námluvám a jsou i méně atraktivní pro samce (Flores et al. 1994; Crews et al. 1998; Rhen & Crews 1999). Samice sice nejsou sterilní, ale sexuálně dospívají v pozdějším věku a tvoří se jim menší počet vitellogenních folikulů, což může mít vliv na reprodukční úspěšnost (Tousignant et al. 1995).

Z tohoto pohledu jsme pro naše experimenty (**Publikace I., II., III., IV. a V.**) během kterých jsme nechtěli testovat vliv inkubační teploty na chování a další life history parametry, zvolili jednotnou inkubační teplotu $28 \pm 0,5$ °C pro druh *E. macularius* a $26 \pm 0,5$ °C pro druh *E. angramainyu*, kde se nám dařilo vylíhnout mláďata.

3.2. Proč hybridizovat?

3.2.1. Hybridizace u ještěřů

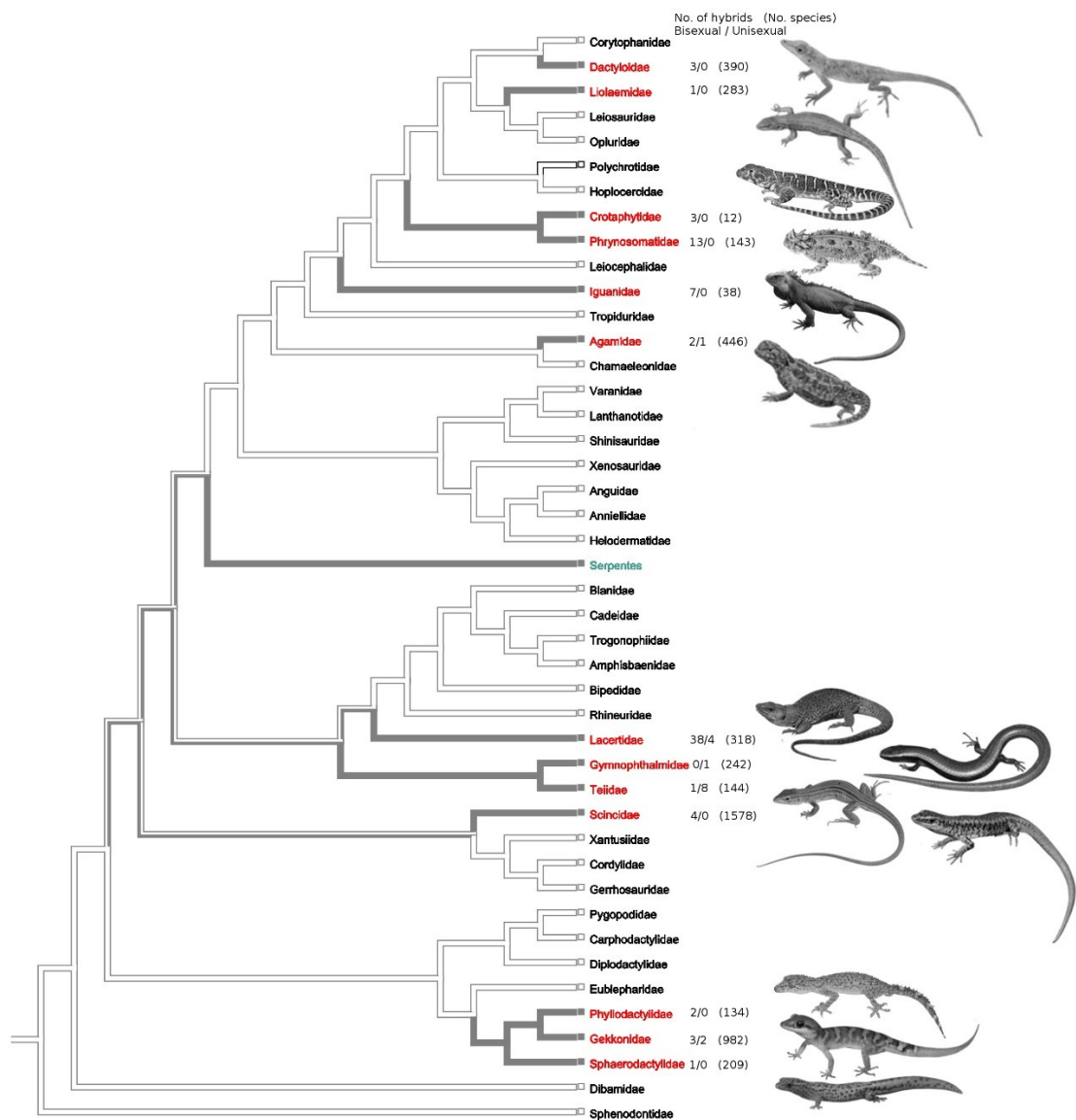
Samotná existence hybridizace, křížení dvou druhů, komplikuje představu o koncepci biologického druhu, která předpokládá, že druhy jsou reprodukčně izolované jednotky (Mayr 1942). Sexuálně množící se druhy jsou pak chráněny tzv. reprodukčně izolačními mechanismy (RIM), které brání toku genů z jednoho druhu do druhého a možného splynutí těchto druhů (Rhymer & Simberloff 1996; Allendorf et al. 2001). Hybridizaci nejčastěji brání postzygotické RIM, které jsou výsledkem genetické odlišnosti druhů a jejich nekompatibility mají za následek neživotaschopnost či sterilitu hybridů (Orr & Presgraves 2000). To vede k selekci a tlaku na vznik tzv. prezygotických RIM, často prekopulačních bariér, které předchází samotné hybridizaci (Hoskin et al. 2005). Zdá se tedy, že hybridizace je pouze chybou v nedostatečně vytvořených reprodukčně izolačních bariérách. To by ale předpokládalo, že hybridizace má pouze negativní dopady na fitness hybridů. Jak se v mnoha studiích už podařilo prokázat, hybridizace může skýtat hybridům i jisté výhody (Grant & Grant 1996; Dowling & Secor 1997; Rieseberg et al. 1999; Pfennig 2007; Stelkens & Seehausen 2009; Baranwal et al. 2012), které je mohou za určitých podmínek zvýhodňovat oproti rodičovským druhům. Zvýšení heterozygotnosti díky křížení může být pro některé ohrožené málo početné druhy jediným řešením, jak se vyhnout problémům plynoucím z inbreedingu (Edmands 2007). Hybridizace má tak svoji nezaměnitelnou roli v evoluci sexuálně se rozmnožujících se druhů napříč všemi taxony díky rychlému generování novinek rekombinací rodičovských genů a jejich neustálého testování přírodním výběrem. Následkem toho může docházet k rychlejší adaptaci na podmínky prostředí, než je tomu běžně při hromadění mutací. To může vést dokonce ke

vzniku nových druhů nebo adaptivní radiaci (Seehausen 2004; Mallet 2007; Genovart 2009; Abbott et al. 2013).

Dobzhansky-Muller model předpokládá, že nekompatibility mezi druhy se hromadí s časem, který uplynul od jejich oddělení, a tudíž roste i síla postzygotických RIM (Dobzhansky 1936; Muller 1942; Orr & Turelli 2001). Nicméně rychlost, s jakou tyto bariéry vznikají, se významně liší mezi hlavními skupinami obratlovců (viz Tabulka 1, **publikace I.**). Ačkoliv se mnoho prací věnovalo teoretické analýze proximálních mechanismů hybridizace, v literatuře poměrně dost chybí přehled empirických studií, a to především u skupiny plazů. To nás vedlo k sepsání první publikace (**publikace I.**), ve které jsme chtěli ukázat stav znalostí o schopnosti ještěřů hybridizovat s důrazem na informace o fitness hybridů (např. životaschopnost, fertilita/sterilita, malformace, kompetice). Předpokládali jsme, že ještěři budou schopni hybridizovat podobně často jako ptáci (Grant & Grant 1992; McCarthy 2006) díky fylogenetické příslušnosti ke skupině Diapsida. Na základě shromážděných výsledků, kdy jsme použili rozdílnost dostupného úseku cytochromu b u rodičovských druhů jako prediktor jejich celkové genetické divergence, se zdá, že ještěři hybridizují i mezi velmi vzdálenými druhy podobně jako je tomu u ryb a žab a dokonce jsou „úspěšnější“ než ptáci, želvy, krokodýli, hadi a savci. Na druhou stranu počet záznamů o hybridizaci ještěřů je oproti jiným skupinám obratlovců poměrně malý, ale hybridizace se vyskytuje napříč jejich fylogenezí (Obr. 3). O vlivu hybridizace na fitness hybridů u ještěřů se neví téměř nic. Většina záznamů o hybridizaci pochází z genetických studií, kde tento typ informací chybí a experimentálně řízené hybridizace, které by parametry fitness podrobně sledovali, jsou velmi ojedinělé (Rykena 2002). Ze zdokumentovaného častého výskyt hybridů ze zpětného křížení či hybridů vyššího řádu u ještěřů, lze usuzovat na alespoň částečnou fertilitu F1 hybridů a tok genů, a to i mezi geneticky velmi vzdálenými rodičovskými druhy, např. *Lacerta schreiberi* x *L. agilis* nebo u *Aspidoscelis tigris* x *A. inornata* za vzniku partenogenetického druhu (Rykena 1996; Dessauer et al. 2000).

Překvapivě se v naší studii (**Publikace I.**) neprokázal vztah mezi genetickou vzdáleností rodičovských druhů a možností toku genů, jak to bylo popsáno v mnoha dříve publikovaných studiích, kde s rostoucí genetickou vzdáleností roste i sterilita a neživotaschopnost hybridů (Edmands 2002; Rykena 2002; Bolnick & Near 2005; Sanchez-Guillen et al. 2014). Vysvětlením může být to, že v našich záznamech je většina hybridů minimálně částečně fertálních a je možné, že existují i hybridy mezi geneticky

vzdálenějšími druhy, kteří už by byli sterilní, ale zatím nebyli objeveni. Nebo těmto hodně geneticky vzdáleným druhům mohou v hybridizaci bránit již vytvořené prekopulační bariéry (odlišné reprodukční chování, jiné preference pro výběr sexuálního partnera apod.) (Heathcote et al. 2016).



Obr. 3 Fylogenetická distribuce hybridizujících druhů v rámci skupiny Squamata, znázorněna je distribuce sexuálních a unisexuálních hybridizací u jednotlivých linií (převzato z **Publikace I.**)

Další naší hypotézou bylo, že ještěři s teplotně určeným pohlavím (TSD) budou schopni hybridizovat navzdory větší genetické odlišnosti snáze, než je tomu u ještěřů s geneticky určeným pohlavím (GSD), kde větší míra sterility se vyskytuje u heterogametického pohlaví s XY nebo ZW chromozomem (Haldane 1922; Presgraves 2010). Například u želv a krokodýlů existují příklady hybridizací mezi fylogeneticky velmi vzdálenými rodičovskými druhy (Karl et al. 1995; Polet et al. 2002). O to větší bylo naše překvapení, že v našem souboru zcela chybí záznamy o hybridizaci u druhů ještěřů s TSD (**Publikace 1**).

Na základě našeho zkoumání o stavu vědění o hybridizaci a jejím vlivu na fitness u ještěřů, jsme nabyli přesvědčení, že naše mnohaletá studie hybridizace u gekončků nemá v odborné literatuře o ještěrech obdoby. Sice jsme proximální mechanismy nechali do velké míry stranou, o to větší úsilí jsme věnovali zachycení všech možných life history parametrů a korelátů fitness, které by nám daly odpovědi, zda hybridizace v tomto případě škodí či prospívá? Tato otázka se může zdát z počátku triviální, ale v kontextu znalostí, resp. neznalostí podobných údajů u ještěřů, je skoro zbytečné se dalšími hypotézami o roli hybridizace v evoluci ještěřů vůbec zabývat.

3.2.2. Hybridizace u gekončků

Aby mohlo dojít k páření u sexuálních druhů, musí být dva jedinci opačného pohlaví ochotných a svolných k tomuto aktu. Aby došlo k úspěšnému páření, je třeba, aby jedinec byl schopný rozpoznat jedince opačného pohlaví a aby došlo k zachování druhu, je třeba rozpoznat jedince vlastního druhu a vyhnout se křížení s cizími. Pro tento účel je zvíře vybaveno smysly, aby mohlo tuto volbu sexuálního partnera provést. Některá zvířata spoléhají spíše na optické signály, jiná na akustické. Velkou skupinou jsou pak signály chemické (např. feromony), které mohou být detekovány několika způsoby: čichovým orgánem, chuťovými pohárky a například u plazů pak pomocí vomeronazálního (Jacobsonova) orgánu (Himstedt 1979; Pough et al. 1998; Cure et al. 2011). Gekoni mají chemorepci značně rozvinutou a využívají ji jak lokalizaci potravy, hledání sexuálního partnera, tak v dalších okruzích chování, jako je antipredační chování nebo kompetice mezi jedinci stejného pohlaví (Schwenk 1993; Dial & Schwenk 1996; Cooper & Steele 1997; Cooper 1998). Mimo tyto signály se živočichové mohou orientovat na základě

vrozeného či imprintovaného prekopulačního chování, které má nalákat potencionálního partnera nebo jej informovat o svých úmyslech. Mezi takové chování patří např. stavba hnízda, nošení „svatebních“ dárků nebo různé epigamní projevy. U námi zkoumaných gekončičků předchází úspěšné kopulaci sled chování, kterými se samec dvoří samici a samice jej buď akceptuje nebo odmítne (viz. Kapitola 3.1). Podobné chování se objevují i u dalších zástupců této čeledi (Greenberg 1943; Kratochvíl & Frynta 2007; Golinski et al. 2015). Při našich experimentech, kdy jsme zkoumali, zda dochází u gekončičků k hybridizaci, jsme se při zjišťování prekopulačních zábran právě zaměřili na schopnost gekončička rozpoznat sexuálního partnera vlastního druhu na základě jeho čichového, resp. vomeronázálního orgánu (četnost olizování vzduchu v blízkosti druhého zvířete a jeho případný zájem o něj) a analýzy prvků epigamního chování (četnost, délka a latence chování). Porovnáním těchto parametrů epigamního chování během hybridizace s pářením rodičovských druhů, jsme pak diskutovali ochotu či neochotu se s jedincem cizího druhu se pářit.

Jelikož gekončičci mají soumravní a noční aktivitu (Seufer et al. 2005), jsou při rozpoznávání sexuálního partnera odkázáni především na čich. U druhu *E. macularius* byla prokázána nejen schopnost gekončička rozpoznávat na základě „očíhávání“ jazykem pohlaví druhého jedince (Mason & Gutzke 1990; Steele & Cooper 1997), ale i schopnost rozpoznat známé a neznámé jedince (LaDage & Ferkin 2006; LaDage & Ferkin 2007). Lze tedy očekávat, že by mohl být schopen rozpoznat i mezi jedinci vlastního a cizího druhu.

Experimenty jsme provedli na dvou úrovních, za první mezi příbuznými, ale geneticky značně vzdálenými druhy, *E. macularius* a *E. angramainyu* a za druhé mezi morfologicky a geneticky odlišnými formami v rámci komplexu druhů/forem kolem *E. macularius*. Druhy i formy mezi sebou liší i ve velikosti a tvaru těla a ve zbarvení (viz. Kapitola 3.1.). Z našich výsledků vyplývá (**Publikace II.**), že samci obou druhů i forem se zajímají a dvoří samicím jak vlastního, tak cizího druhu a samice jsou ochotné se s nimi pářit. Nicméně, samci i samice se začnou o potenciálního partnera zajímat dříve (první přiblížení a oliznutí vzduchu směřované k druhému zvířeti), pokud jde o jejich vlastní druh než o cizí. Je možné, že pro tuto diskriminaci využívají jiné než pachové signály. Například jejich preference sexuálního partnera může být ovlivněna jeho velikostí. V některých studiích se prokázalo, že samice nebo i samec může preferovat větší jedince před menšími (Andersson & Iwasa 1996). Na druhou stranu evoluce velikosti těla není jen pod sexuálním selekčním tlakem, ale je funkcí i klimatu, dlouhodobé potravní nabídky, predačním tlakem

nebo kompetitivními interakcemi (Nosil et al. 2005). Preference většího partnera se ale v naší studii neprokázala, protože celkový čas, kdy jevíli zájem o druhé zvíře, byl pro obě pohlaví podobný, jak při interakci s jedincem vlastního, tak cizího druhu. Ochotu pářit se s menšími jedinci lze vysvětlit tím, že poměr menšího a většího druhu odpovídal poměru ve velikosti, jaký se vyskytuje u skupiny Squamata mezi sexuálně dospělým jedincem a jedincem o maximální velikosti (Shine & Charnov 1992). Tento velikostní poměr mezi partnery je podobný typickému velikostnímu poměru mezi hybridizujícími ještěry (**Publikace I.**). Gekončici tedy mohou vnímat menší druh/formu jako sexuálně dospělého jedince vlastního druhu, a proto jsou ochotní se s ním pářit. Pro samici, díky její vyšší investici do reprodukce, by mohl být výběr partnera důležitější. Je však možné, že jejich abundance v přírodě je nízká, a proto nemají tolik příležitostí k výběru sexuálního partnera. Pokud je samice ve správné ovulační fázi, výběr partnera možná neřeší (Kratochvíl & Frynta 2007). V některých případech může samice naopak volit raději heterospecifického sexuálního partnera (Veen et al. 2001; Pfennig 2007). Další vysvětlením může být to, že samice se páří opakovaně s různými samci a výběr probíhá až uvnitř samice, jako například kompetice spermií nebo skrytá samičí volba (Madsen et al. 1992; Olsson & Madsen 1998; Simmons 2005), přičemž samice uchovávají spermie po celou reprodukční sezónu (LaDage et al. 2008). Výjimečně mohou samice uchovat spermie i do začátku druhé sezóny. Samci obou druhů se dále nelišili ani době, kdy vibrovali ocasem na svoji nebo cizí samici. Jediný rozdíl byl, že samec *E. angramainyu* okusoval vlastní samici kratší dobu než cizí. Myslíme si s ohledem na zvýšenou četnost kousnutí samce při jeho odmítnutí u druhu *E. angramainyu*, vede samce u vlastních samic k větší obezřetnosti. Vyšší agresivita při odmítnutí u většího druhu by odpovídala výsledkům vlivu velikosti druhu na zvolenou strategii při antipredační reakci u dvou druhů gekonů rodu *Teratoscincus*. Větší druh *T. keyserlingii* volí při antipredační reakci na taktilní stimul častěji vysoký postoj a útok než menší druh *T. scincus* (**Příloha 1**). Etologie antipredačního chování a chování samice při odmítnutí samce je podobná u všech zmíněných druhů/forem (**Publikace V.**). Alternativním vysvětlením může být ale fakt, že samice *E. angramainyu* celkově odmítaly (útěk, příkřčení se zdviženým vlnicím se ocasem) samce delší dobu než samice *E. macularius*, tudíž zvýšený výskyt útoků na samce může být jen eskalací odmítnutí. Nereceptivita těchto samic by i vysvětlovala, proč samec *E. angramainyu* okusoval více cizí samice, které mohly být jen více receptivní k páření. Celkové analýzy prvků chování sice ukázaly, že samice ale i samci během interakce s vlastním a cizím druhem se chovají trochu jinak, ale to jim nakonec

nebrání v obou případech se spářit. Úspěšnost páření je možná nejvíce ovlivněno receptivitou samice, a to je dáno její fyziologií než její preferencí sexuálního partnera vlastního druhu.

Zjistili jsme, že mezi druhy *E. angramainyu* a *E. macularius* ani mezi „tmavou“ a „žlutou“ formou druhu *E. macularius* nejsou vytvořeny dostatečné prekopulační bariéry, které by zabránily křížení. V další fázi nás zajímalo, jestli mezi geneticky fylogeneticky vzdálenějšími druhy *E. macularius* a *E. angramainyu* jsou vytvořeny postzygotické bariéry, které by bránily vzniku F1 hybridů a dalšímu toku genů mezi druhy (vnik F2 hybridů a hybridů ze zpětného křížení s rodičovskými druhy) (**Publikace III. A IV.**). K tomu jsme využili srovnávací analýzy různých life history parametrů hybridů a jejich rodičovských druhů (hmotnost vajíčka, líhnivost, hmotnost mláděte po vylíhnutí a jeho růst do dospělosti, fertilita, morfologické abnormality). Cílem bylo zjistit, zda mají hybridi horší fitness než rodičovské druhy nebo naopak lepší, například díky heteroze (Edmands 1999; Baranwal et al. 2012).

Ukázalo se, že navzdory poměrně velké genetické odlišnosti těchto dvou druhů (22 % HKY85 distance úseku cytochromu b dlouhého 303 bp, Palupčíková et al. nepublikovaná data) jsou samice druhu *E. macularius*, které se zkřížily se samcem *E. angramainyu*, schopné snášet oplozená vejce. Tato vejce však měla jen zhruba poloviční líhnivost, než má běžně druh *E. macularius*. Na druhou stranu byla úspěšnost líhnutí ale srovnatelná se vzácnějším druhem *E. angramainyu*. Přežívání těchto F1 hybridů bylo vysoké a srovnatelné s rodičovskými druhy. Ačkoliv jejich velikost po vylíhnutí spíše odpovídala mláďatům menšího druhu *E. macularius*, v dospělosti dorostli větší velikosti a fenotypově se spíše přiblížili většímu druhu *E. angramainyu* (Obr. 1, 4 a 5 v **Publikaci III.**). Tato nesrovnalost by napovídala, že ačkoliv jsou F1 hybridi geneticky nositeli znaku pro větší velikost zděděnou po otci, na počátku je jejich velikost dána matkou, tedy menším druhem *E. macularius*. Velikost vajíčka je u ještěřů limitována velikostí samice, resp. velikostí její břišní dutiny (Kratochvíl & Frynta 2006; Kratochvíl & Kubička 2006). Větší velikosti druh/forma nedosáhne zvětšováním rychlosti růstu, ale prodloužením času, kdy exponenciálně roste. F1 hybridi tak dosáhnou dospělé velikosti, kdy jsou schopni se reprodukovat, rychleji než druh *E. angramainyu* (**Publikace IV.**). Za určitých podmínek to může znamenat pro hybridy značnou výhodu oproti rodičovskému druhu (Pfenning 2007).

Ukázalo se, že většina těchto F1 hybridů jsou fertlních a samice nejsou horší v produkci vajec, resp. jsou srovnatelné s druhem *E. macularius* (Tab.1. v **Publikaci III.**). To platí i pro produkci vajec samicemi druhu *E. macularius*, pokud se zkřížili s F1 hybridem. Výrazný rozdíl jsme pak našli ale v líhnivosti těchto vajec. Líhnivost vajec hybridů křížených zpětně s rodičovským druhem *E. macularius* byla úspěšná oproti křížení s druhem *E. angramainyu*, kde se nevylíhlo jediné mládě. Tato asymetrie v úspěchu zpětného křížení byla pozorována také např. u ryb (Bolnick et al. 2008), obojživelníků (Arnold et al. 1996; Devitt et al. 2011), ještěřů (Rykena 2002; Robbins et al. 2014), nebo u hmyzu (Sanchez-Guillen et al. 2012). Mláďata F2 hybridů se líhla velmi špatně (líhnivost pouze 6 %) a všechna čtyři mláďata měla zjevné deformace v oblasti ocasu. Jen jedno mládě se dožilo dospělosti. (Obr. 1. v **Publikaci III.**). Tyto deformace se u jiných typů hybridů nevyskytovaly. Dá se říci, že problémy v líhnivosti nejsou dány sterilitou F1 hybridů, ale spíše nekompatibilitou genů při rekombinaci v rané fázi vývoje ve vajíčku. V evoluci není až tak významné, jestli vznikne F1 hybrid, pokud je sterilní, ale právě významná je až schopnost F1 hybridů je dál se rozmnožovat, což je u gekončků umožněno právě přes úspěšnost při zpětném křížení s druhem *E. macularius*. To umožňuje introgresi genů *E. angramainyu* do genotypu běžnějšího druhu *E. macularius*.

Ztráta viability vyžaduje dvakrát více času, který dělí hybridizující druhy, než ztráta fertility např. u ryb (Bolnick & Near 2005) nebo ptáků (Price & Bouvier 2002). Naše výsledky o fitness a fertilitě gekončků, navzdory jejich genetické odlišnosti dle cytochromu b, jen potvrzují naše domněnky o možnosti většího výskytu úspěšných hybridizací u ještěřů, než je doposud známo (**Publikace I.**). Hybridizující gekončci, *E. macularius* a *E. angramainyu*, na základě genetických rozdílů na cytochromu b (metodika v **Publikaci I.**) se totiž řadí na pomyslnou první příčku v Tabulce 2 v **Publikaci I.** Toto zjištění je o to zajímavější, že se jedná o první záznam hybridizace mezi druhy s teplotně určeným pohlavím (TSD) u ještěřů. Naše hypotéza, že druhy s TSD budou snáze hybridizovat, díky absenci pohlavních chromozomů, však vyžaduje větší množství hybridizujících druhů jak s TSD, tak s GSD, jejichž rodičovské páry budou srovnatelně geneticky odlišní.

Co ale můžeme říct je, že F1 hybridi se na základě morfologických analýz a růstových parametrů odlišili od obou rodičovských druhů (**Publikace III. a IV.**). Tyto nové transgresivní fenotypy se vyskytují u hybridů poměrně často (Stelkens & Seehausen

2009; Dittrich-Reed & Fitzpatrick 2013; Hladlovská et al. 2013; Nichols et al. 2015) a v některých případech umožňují hybridům například obsadit nové biotopy či rozšířit potravní niku (Grant & Grant 1996) nebo být úspěšnější proti predátorům a obhajování teritoria než rodičovské druhy (Robbins et al. 2010). Díky hybridizaci tak vznikají poměrně snadno nové kombinace znaků, které mohou usnadnit adaptivní radiaci (Dowling & Secor 1997; Seehausen 2004) a nabízí stále nové a nové evoluční scénáře, které jsou neustále testovány přírodním výběrem.

3.3. Proč měnit antipredační strategie během ontogeneze?

Přežití je mimo jiné velmi ovlivněno vhodně zvolenou antipredační strategií. Někdy je lepší utéct, jindy se vyplatí pokusit se predátora zastrašit či zmást (Medill et al. 2011). Ukázalo se, že antipredační chování je kromě vnějších podmínek ovlivněno také např. velikostí, kondicí, zkušeností, zbarvením, mírou predáčního tlaku, ale také stářím zvířete a jeho fyziologickým stavem a možnostmi (Marcellini & Jenssen 1991; Van Buskirk & Schmidt 2000; Benard 2004; Dangles et al. 2007). Úspěšnost a volba strategie v těchto interakcích s predátorem ovlivňuje fitness zvířete (Lind & Cresswell 2005). Jelikož se během ontogenetického růstu mění různé morfologické a fyziologické vlastnosti jedince, pro mláďata a dospělé se selekční tlaky mohou výrazně lišit (Pough 1978; Garland 1985; Irschick 2000; Herrel et al. 2006). Na základě toho se pak může v ontogenezi měnit i chování a preference jedinců (Law 1991; Lind & Welsh 1994; Keren-Rotem et al. 2006; Eskew et al. 2009).

U plazů jsou různé antipredační strategie často spojené se změnou zbarvení (Pough 1976; Fresnillo et al. 2016). Například mláďata štíhlovky americké *Coluber constrictor*, oproti jednobarevnému zbarvení dospělců, mají kryptické skvrnitě zbarvení. Pokud jsou konfrontovány predátorem jsou významně agresivnější než dospělí jedinci. Ti volí raději útek (Creer 2005). U ještěrek *Acanthodactylus beershebensis* mají mláďata výrazný modrý ocásek, který jim umožňuje odlákat pozornost predátora od životně důležitějších částí těla. V tomto věku se mnohem častěji při lovu potravy zdržují v otevřených habitatech, kde je riziko predace vyšší. Dospělci, kteří jsou zbarvení celí krypticky, jsou mnohem zdrženlivější (Hawlena et al. 2006).

U gekončíka nočního *E. macularius* jsme chtěli studovat jeho schopnosti rozpoznávat různé typy hadích i jiných obratlovčích predátorů (Landová et al. 2016, Suchomelová et al. nepublikováno). V první řadě nás zajímal etogram antipredačních reakcí a zda se toto chování může měnit v závislosti na věku, velikosti, kondici a zbarvení jedince (**Publikace V.**). K tomu jsme použili devět věkových skupin gekončků od narození po dospělé jedince a útok predátora jsme simulovali rozprašovačem s vodou a taktilně vatovou tyčinkou šroucháním do oblasti sakrální oblasti hřbetu. U gekončků jsou mláďata výrazně zbarvena, kdy se střídají tmavé a bílé až sytě žluté pruhy (viz. Obr. 1 v **Publikaci V.**). Toto zbarvení přetrvává zhruba do věku 70 až 90 dní. Pruhovaný vzor se postupně rozpadá, ale u subadultních gekončků ve věku 90 až 450 dní lze pruhovaný charakter ještě rozpoznat. Na začátku tohoto období jsou u samců už patrné hemipenisy. Plně vybarvení jsou dospělci individuálně ve věku minimálně 150 dní, ale spíše v pozdějším věku. Reprodukční dospělosti dosáhnou ve věku kolem 280 až 350 dnech a ví se, že tato doba je ovlivněna inkubační teplotou, ve které se gekončici líhli, sezónou a individuální variabilitou (Tousignant et al. 1995; Sakata & Crews 2004).

Sledovali jsme tři okruhy reakcí: obranné postoje, vokalizace občas spojené i s útokem a útek. Postoje mohou být jak tzv. vysoké, kdy zvíře má napnuté končetiny a snaží se být co největší nebo naopak ztuhne přimknutý k substrátu. Během postoje zvíře může krátce vibrovat ocasem v substrátu nebo jej zvedne od země a pomalu s ním vlnivě pohybuje ze strany na stranu. Tyto postoje jsou podobné s odmítavým chováním nereceptivní samice během námluv (**Publikace II.**).

Z našich výsledků vyplývá, že strategie antipredačního chování se s věkem mění. V raném věku mláďata moc neutíkají a místo toho se snaží predátora, v tomto případě experimentátora s vatovou tyčinkou a rozprašovačem, zastrašit vřeštěním (vokalizací) se silně rozevřenou tlamou. Toto chování se s věkem postupně vytrácí a místo toho začnou gekončici převážně utíkat (Obr. 2 a 3 v **Publikaci V.**). Proč malá mláďata volí tuto zastrašovací strategii může vysvětlit možná jejich velikost a s ní spojená jistá fyziologická omezení, jež mají dopady na jejich performanční schopnosti. Například malá mláďata užovky *Natrix sipedon* mají oproti dospělcům sníženou vytrvalostní kapacitu (Pough 1978) nebo u agamy rodu *Stellio* (*Stellagama*) nebo u hatérií *Sphenodon punctatus* s rostoucí velikostí roste i absolutní rychlost sprintu (Huey & Hertz 1982; Nelson et al. 2006). Toto vysvětlení by bylo v souladu s výsledky u gekončků, kdy větší jedinci v rámci svojí věkové kategorie volili častěji útek než ti menší (**Publikace V.**).

Alternativním vysvětlením změny antipredační strategie je změna zbarvení z kontrastního mláděcího vzoru na skvrnitý vzor dospělého gekončíka, který na kamenitém podkladu, může působit krypticky (Marcellini 1977; Ruxton et al. 2004). Jelikož u gekončíka *E. macularius* existuje poměrně velká variabilita zbarvení (Seufer et al. 2005) a zároveň obývají poměrně širokou niku habitatů, lze se domnívat, že tato variabilita by mohla souviset se selekčním tlakem na vhodné kryptické zbarvení pro konkrétní lokalitu. Podobnou útěkovou antipredační strategii volí i ropušníci rodu *Phrynosoma* s kryptickým zbarvením, pokud jsou odhaleni predátorem (Sherbrooke 2008; Cooper & Sherbrooke 2010). Kontrastní pruhované zbarvení mlád'at v kombinaci s vokalizací by mohlo být spíše výstražným signálem nebo dokonce by mlád'ata mohla mimetizovat některé jedovaté sympatrické hady, např. druh *Bungarus caeruleus* (Elapidae). Podobně mimetizují mlád'ata ještěrky *Heliobolus (Eremias) lugubris* zbarvením a pohyby brouka rodu *Anthia* (Carabidae), který vypouští dráždivou tekutinu při obraně (Huey & Pianka 1977). Alternativně může pruhované zbarvení být optickým vnitrodruhovým signálem, který má zabránit případné infanticidě jako například červené zbarvení u mlád'at druhu *Acanthodactylus erythrurus* (Fresnillo et al. 2015).

V potaz musíme brát i rozdílný predací tlak na mlád'ata a dospělé, jelikož mládě má širší okruh potencionálních predátorů než větší dospělec (Head et al. 2002). To vytváří silnější selekci na vhodně zvolenou antipredační strategii. Ukazuje se, že mlád'ata ještěrů jsou ochotna častěji riskovat než dospělci (Samia et al. 2016). Jelikož mlád'ata nedisponují velkými energetickými zásobami (např. v ocase) a u hmyzožravých druhů mohou lovit jen odpovídající velikost hmyzu, jsou nucena strávit více času sháněním potravy než dospělí jedinci. A to i za cenu vyššího rizika predace (Daniels 1984; Hawlena et al. 2006; Samia et al. 2016).

4. Závěry

Selekce způsobená predací, stejně jako chybné rozhodnutí pářit se s nepříbuzným druhem, kladou nároky na různá přizpůsobení živočichů ve smyslu morfologických, behaviorálních a kognitivních adaptací. V některých případech, jako například u skupiny Squamata, kam patří i naše studované druhy gekončků, je frekvence takovýchto chyb v rozpoznání partnera vlastního a cizího druhu poměrně častou záležitostí. Je zajímavé, že schopnost vytvářet plodné hybridy není z pohledu genetické vzdálenosti (rodičovských druhů) vázána pouze na partenogenetické druhy, ale vyskytuje se i u geneticky velmi distantních (20%) sexuálních druhů. Jednu z nejvyšších distancí mezi rodičovskými druhy, kde k produkci plodných hybridů dochází najdeme právě u našeho experimentálního modelu vzdálené hybridizace mezi *E. macularius* a *E. angramainyu*. Rozdíly v sexuálním chování mezi nimi sice najdeme (především u samic), ale další hybridizaci to nebrání. Další osud hybridů, nejen těch vzdálených, pak závisí na spoustě parametrů. Řada z nich souvisí s tělesnou velikostí a rychlostí růstu. Reprodukční možnosti hybridů jsou sice omezené, ale díky úspěšnému zpětnému křížení může docházet k dalšímu toku genů. Hybridy pak mají nejen intermediární velikost, ale i rychlost růstu. Větší velikost hybridů oproti jednomu rodičovskému druhu/formě pak může hybridům poskytnout jisté výhody. V předchozích pracích bylo již publikováno, že větší gekončci mají výhodu ve vnitropohlavních interakcích a také mohou zřejmě používat odlišné antipredační strategie. Ukázali jsme totiž jasně odlišné antipredační strategie používané malými mláďaty a dospělými jedinci. Dospělí gekončci mohou využít svoji absolutně větší tělesnou velikost při preferovaném útěku. Potencionální výhody a nevýhody obou procesů závisí na věku jedinců (predace a s ní související rychlost růstu), morfologické odlišnosti (tělesná velikost je klíčová pro mnoho dalších procesů), genetické odlišnosti či odlišnosti v sexuálním chování.

Dále shrnuji hlavní výsledky této práce v bodech, jež odpovídají na otázky kladené v cílech práce:

- Ještěři hybridizují i mezi velmi vzdálenými druhy podobně jako je tomu u ryb a žab a dokonce jsou „úspěšnější“ (tj. hybridizují geneticky vzdálenější rodičovské druhy) než ptáci, želvy, krokodýli, hadi a savci.

- Počet záznamů o hybridizaci a o vlivu na fitness hybridů u ještěřů je oproti jiným skupinám obratlovců poměrně malý, ale hybridizace se vyskytuje napříč celou jejich fylogenezí.
- Překvapivě se v naší studii neprokázal vztah mezi genetickou vzdáleností rodičovských druhů a sterilitou anebo neživotaschopností hybridů u ještěřů. Alespoň částečná fertilita F1 hybridů a tok genů se vyskytoval i u příkladů hybridizací mezi geneticky nejvzdálenějšími rodičovskými druhy.
- Zjistili jsme, že mezi druhy *E. angramainyu* a *E. macularius* a ani mezi „tmavou“ a „žlutou“ formou komplexu druhu *E. macularius* nejsou vytvořeny dostatečné reprodukční bariéry, které by zabránily křížení.
- Mezidruhovému křížení druhů *E. angramainyu* a *E. macularius* je prvním záznamem o křížení u druhů s teplotně určeným pohlavím (TSD) u ještěřů a zároveň patří k hybridizaci mezi geneticky nejvzdálenějšími druhy ještěřů ve srovnání s dostupnou literaturou.
- Na základě morfologických analýz a analýzy zbarvení a růstových parametrů se liší jak rodičovské druhy *E. angramainyu* a *E. macularius*, ale také jejich F1 hybridů.
- F1 hybridů jsou životaschopní a fertilitní a introgrese (tok genů) druhu *E. angramainyu* do genomu *E. macularius* je umožněna díky zpětnému křížení. Mezidruhoví hybridů, s výjimkou F2 hybridů, nemají žádná poškození a ani horší životaschopnost nebo růstovou dynamiku.
- Lze očekávat, že ještěři hybridizují častěji, a i mezi geneticky rozdílnějšími druhy, než se dodnes ví.
- Gekončící noční mění antipredační strategii během ontogeneze. Mláďata jsou odvážnější, volí spíše zastrašování postojem, vokalizací a častěji útočí. Dospělí gekončící volí útek. Tato změna chování je doprovázena změnou zbarvení od kontrastního pruhování mláďat po kryptické skvrnitě zbarvení dospělců.

5. Literatura

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6. Publikace

Seznam příložených prací / Papers attached to this thesis

- I. Jančúchová-Lásková, J.**, Landová, E. & Frynta, D. (2015) Are genetically distinct lizard species able to hybridize? A review. *Current Zoology* 61: 155-180.
- Koncipoval a navrhl experiment/Conceived and designed the experiments: DF, EL, JJJ; shromáždil data/collected the dataset: JJJ; analyzoval data/analysed the data: DF, JJJ; psal článek/wrote the paper: DF, JJJ, EL.
- II.** Landová, E., **Jančúchová-Lásková, J.**, Kratochvíl, L., Polák J. & Frynta, D.: Divergence in sexual behaviour during distant and close hybridization in eublepharid geckos: Experimental crossing of *Eublepharis macularius* and its two congeners. (manuscript)
- Koncipoval a navrhl experiment/Conceived and designed the experiments: EL, DF, JJJ; idea/developed the idea: LK; provedl experiment/performed the experiments: JJJ; analyzoval data/analysed the data: EL, JJJ, DF; psal článek/wrote the paper: EL, JJJ, DF, JP.
- III. Jančúchová-Lásková, J.**, Landová, E. & Frynta, D. (2015) Experimental crossing of two distinct species of leopard geckos, *Eublepharis angramainyu* and *E. macularius*: viability, fertility and phenotypic variation of the hybrids. *Plos One* 10.
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- IV.** Frynta, D., **Jančúchová-Lásková, J.**, Frýdlová, P. & Landová, E.: Fast or slow? A comparative study of body weight trajectories in three species of the genus *Eublepharis* and their hybrids. (manuscript)

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- V. Landová, E., **Jančúchová-Lásková, J.**, Musilová, V., Kadochová, S. & Frynta, D. (2013) Ontogenetic switch between alternative antipredatory strategies in the leopard gecko (*Eublepharis macularius*): defensive threat versus escape. Behavioral Ecology and Sociobiology 67: 1113-1122.

Koncipoval a navrhl experiment/Conceived and designed the experiments: EL, DF; provedl experiment/performed the experiments: ŠK, VM, JLL; analyzoval data/analyzed the data: DF, JLL; psal článek/wrote the paper: EL, DF, JLL.

Souhlas korespondenčních autorů jednotlivých prací s podílem Mgr. Jitky Jančúchové Láskové na manuskriptech a publicích zařazených do této disertační práce, tak jak je vyjádřen výše v seznamu příložených prací.

RNDr. Eva Landová, Ph.D.

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Doc. RNDr. Daniel Frynta, Ph.

I.

Are genetically distinct lizard species able to hybridize? A review

Jitka Jančúchová-Lásková, Eva Landová, Daniel Frynta

Current Zoology (2015) 61:155-180

Are genetically distinct lizard species able to hybridize? A review

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Abstract Animal species are delimited by reproductive isolation mechanisms (RIMs). Postzygotic RIMs are mainly products of genetic differences and thus their strength increases with elapsed divergence time. The relationship between postzygotic reproductive isolation and genetic divergence, however, differs considerably among major clades of vertebrates. We reviewed the available literature providing empirical evidence of natural and/or experimental hybridization between distinct species of lizards (squamates except snakes). We found that hybridization events are widely distributed among nearly all major lizard clades. The majority of research focuses on parthenogenetic species and/or polyploid hybrids in families Lacertidae, Teiidae and Gekkonidae. Homoploid bisexual hybrids are mainly reported within Lacertidae and Iguania groups. As a proxy of genetic divergence of the hybridizing taxa we adopted nucleotide sequence distance (HKY85) of mitochondrial *cyt b* gene. The upper limit of genetic divergence was similar with regard to both parthenogenetic and bisexual hybrids. Maximum values of these distances between hybridizing species of lizards approached 18%–21%, which is comparable to or even exceeds the corresponding values reported for other principal clades of vertebrates. In spite of this, F₁ hybrids are typically at least partially fertile in lizards and thus genetic introgression between highly divergent species is possible. The relationship between the genetic distance and hybrid fertility was not found [Current Zoology 61 (1): 155–180, 2015].

Keywords Hybridization, Introgression, Fertility, Viability, Genetic divergence, Lizards

Hybridization may be defined as “interbreeding of individuals from what are believed to be genetically distinct populations, regardless of the taxonomic status of such populations” (Rhymer and Simberloff, 1996). Currently, this process has become an important issue in conservation biology (Allendorf et al., 2001). Anthropogenic effects like translocations and habitat modifications facilitate breaking of the natural barriers between genetically distinct populations and/or species. The increased rates of hybridization have some harmful effects sometimes even resulting in extinctions (Rhymer and Simberloff, 1996; Wolf et al., 2001). In contrast to this, the evidence of outbreeding depression is scarce (Edmands, 2007) and, in the past, natural hybridization events may have been really important in the evolution of many plant and animal species, especially during the speciation processes and the emergence of adaptive characters (Mallet, 2007; Genovart, 2009; Abbott et al., 2013). The distinction between species and/or populations that have arisen through natural and anthropogenic hybridization is sometimes difficult. One such example may be represented by the deep divergences in the mitochondrial lineages and their incongruence with nuclear

markers in the endangered South Asian turtles of the genus *Mauremys* (Fong et al., 2007, Somerová et al., in print). Moreover, interbreeding of distinct populations is sometimes the only available way how to avoid inbreeding depression which is becoming an increasingly important cause of decline in endangered species (cf. Miller et al., 2009 for tuatara). Thus, an adequate taxonomic and genetic delimitation of the conservation units is a crucial problem of the conservation policy (Frankham et al., 2009).

Species of sexually reproducing organisms are delimited by prezygotic and/or postzygotic reproductive isolation mechanisms (RIMs). The prezygotic reproduction barriers cause either the premating isolation (e.g., due to different female preferences and different mating behavioral patterns) or the postmating gametic incompatibilities that may be caused by reduced sperm survival in interspecific crosses or through incompatibilities between sperm proteins and egg receptors (examples are reviewed in Servedio, 2001).

The postzygotic RIMs result mainly from the genetic divergence and these consequent incompatibilities cause inviability or sterility of the hybrids (Orr and Presgraves,

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2000; Coyne and Orr, 2004). This makes the avoidance of interbreeding advantageous and further enhances the evolution of the prezygotic, mostly precopulatory, RIMs by reinforcement (Hoskin et al., 2005).

Recently, Matute et al. (2010) demonstrated in two pairs of *Drosophila* species that the number of genes involved in postzygotic isolation increases with the square of the sequence divergence between the hybridizing species. This conforms to the Dobzhansky-Muller model (Dobzhansky, 1936; Muller, 1942) predicting that the incompatibilities are cumulative and the strength of the reproductive isolation increases with elapsed divergence time (Orr and Turelli, 2001). The relationship between postzygotic reproductive isolation and genetic divergence was reported in many studies (e.g., Ayala, 1975; Coyne and Orr, 1989; 1997; 2004; Sasa et al., 1998; Price and Bouvier, 2002; Bolnick and Near, 2005; Sánchez-Guillén et al., 2014). However, the rate of the formation of reproductive isolation barriers differs significantly among major vertebrate clades.

While mammalian species typically lose their ability to form F_1 hybrids after two million years of independent evolution, the cases of successful hybridization of species separated by dozens of million years were reported in teleost fishes, birds and turtles (Wilson et al., 1974; Prager and Wilson, 1975; Karl et al., 1995; Fitzpatrick, 2004; Bolnick and Near, 2005; see Table 1).

When discussing the ability of phylogenetically distant animals to still produce at least viable F_1 hybrids, we can consider the role of the genetic vs. temporal divergence. Many studies show that the mutation rates in various organisms are fundamentally different (Hughes and Mouchiroud, 2001; Edmands, 2002; Ho et al., 2005; Hedges et al., 2006; Jiang et al., 2007; Nabholz et al.,

2009; Eo and DeWoody, 2010; Bromham, 2011; Shaffer et al., 2013). As the elapsed time is a function of both the genetic divergence and the clade specific mutation rate, the period of phylogenetic isolation itself is not suitable for comparative purposes. From this perspective, the divergence of the genome is a better predictor for the estimation of the limits of hybridization and genetic introgression (Galtier et al., 2009).

In recent years, theoretical aspects of evolutionary mechanisms of hybridization have been a focus of many reviews (Seehausen, 2004; Mavárez et al., 2006; Mallet, 2007; Mavárez and Linares, 2008; Barton et al., 2009; Fitzpatrick, 2012; Abbott et al., 2013; Bailey et al., 2013; Barton, 2013; Dittrich-Reed and Fitzpatrick, 2013; Eroukhanoff et al., 2013; Sætre, 2013). Despite of the numerous papers devoted to the theoretical analysis of the proximate mechanisms of hybridization there have been scarce reviews of empirical hybrid studies, especially those concerning reptiles.

Squamates, namely lizards, are the most species-rich clade (5,947 lizard species according to The Reptile Database Uetz and Hošek, 2014) of extant taxa traditionally referred to as reptiles. The phylogenetic position of the squamates as a sister group of the archosaursian clade (including both birds and crocodylians; Pough et al., 2005) and the knowledges concerning their ability to hybridize being crucial for the interpretation of the previously reported sharp differences between mammals and birds in this respect (Fitzpatrick, 2004). The potential ability of genetically divergent species of lizards to hybridize would support the view that such an ability previously reported in birds is not an evolutionary novelty, but rather an ancestral quality of at least the entire Diapsida clade (cf. Li and Lecointre, 2009).

Table 1 Reported cases of record holders that produce viable hybrids despite their long time of separation in various lineages based on the published articles

Lineage	Family	Time of divergence (million years ago)	References
Fishes	Lepisosteidae	33–100	Hedges et al., 2006; Herrington et al., 2008.
	Centrarchidae	35	Bolnick and Near, 2005.
Frogs	Hylidae	22–80	Karl et al., 1995; Smith et al., 2005.
Lizards	Iguanidae	10–20	Rassmann, 1997.
Snakes	Pythonidae	35	Hoser, 1988; Rawlings et al., 2008.
	Colubridae	30	Hedges et al., 2006; LeClere et al., 2012.
Turtles	Cheloniidae	50–63	Karl et al., 1995; Naro-Maciel et al., 2008.
Crocodiles	<i>Crocodylus</i>	10	Polet et al., 2002; Brochu, 2003.
Birds	Anatidae	28	Gonzalez et al., 2009.
Mammals	Balaenopteridae	5–8	Hedges et al., 2006; Glover et al., 2013.
	Delphinidae	8	Hedges et al., 2006; Zhang et al., 2014.

The empirical evidence about the limits of the hybridization ability and costs associated with outbreeding is also urgently required for the conservation practice. Traditionally, hybridization between distinct populations has been interpreted exclusively as a threat to the genetic assimilation, especially for the population which are smaller and/or competitively inferior. A deliberate introduction of the green iguanas *Iguana iguana* on the Guadeloupean Archipelago resulted in heavy costs for the population of the rare endemic *Iguana delicatissima*. In this particular case, successful hybridization led to introgression and strong reproductive competition (Breuil, 2000). Introduction of the widespread iguanid lizard *Ctenosaura similis* to the Uvilla Island inhabited by the critically endangered *C. bakeri* resulted in only a limited introgression (Pasachnik et al., 2009). Currently, Robbins et al. (2010, 2014) reported that natural hybrids of *Sceloporus woodi* and *S. undulatus* exhibit transgressive phenotypes. This finding highly facilitates a genetic introgression, which has fairly positive effects on the fitness of the interbreeding species. Thus, the role of hybridization for conservation is not exclusively negative.

We felt a review of empirical hybrid studies in lizards would have prudent and informations about the potencial consequences of hybridization ability substantial for conservation. In this paper, we reviewed well-documented empirical cases of natural and/or artificial hybridization between distinct species and/or races of lizards. We did not speculate about the proximate mechanisms of hybridization and speciation, and instead showed the status of empirical knowledge concerning the ability of lizard species to hybridize. We listed pairs of parental species reported to produce hybrids of the first filial generation or higher order hybrids and we explored the limits of between-species hybridization and introgression.

Hybridization success is constrained by proximate mechanisms related to the genetic divergence of the hybridizing parental species. The genetic divergence may be viewed as best surrogate of the evolutionary distance and also the best currency for comparative studies of hybridization (Edmands, 2002). Because the entire genomes and even sequences of multiple nuclear genes are only available in a few model species of reptiles (Organ et al., 2008; Janes et al., 2010), we rely on mitochondrial genes, which are available for the majority of the concerned taxa. Thus, we utilized the sequence divergence of the mitochondrial cytochrome *b* gene and treated these values as a proxy of genetic divergence

between the parental species.

This approach was previously successfully applied to assess whether genetic divergence predicts reproductive isolation of damselflies (Sánchez-Guillén et al., 2014). Also, recent demonstration of the mutation rates of the mitochondrial genes predicting speciation rates and diversification in sauropsid lineages (Eo and DeWoody, 2010) strongly substantiates the usage of the mitochondrial *cyt b* gene divergence as a proxy of genetic divergence that may constrain hybridization. However, the use of mtDNA is further complicated by the fact that sexual selection operating on males is not properly reflected by maternal genes and the male-based gene flow is not reflected in these data. We excluded snakes from our analyses because their mitochondrial genome includes a duplicated control region, which may confuse the ratio between the substitution rates of the mitochondrial and nuclear genes (Jiang et al., 2007).

1 Materials and Methods

We collected as many instances of hybridization in lizards (Squamata without snakes) as we could find. The search of literature was performed in two steps. Since 2005 to 2006 we performed a broad search of literature that included scientific databases Web of Science (<https://apps.webofknowledge.com/>), Biological Abstracts (<http://thomsonreuters.com/zoological-record/>) and Zoological Record (<http://thomsonreuters.com/biological-abstracts/>). Information from other literature sources (coming from amateur herpetologists) was also included. We gathered available information about the distribution of hybridization in lizards, viability and/or fertility of the hybrids and also the occurrence of the parthenogenetic hybrid species. The only criterion was the reliability of the specific information. The second search was performed since January to November 2014 using only the Web of Science. We searched for the keywords: hybrid* AND reptile; hybrid* AND the name of the lizard family; hybrid* AND lizard*; parthenogen* AND lizards. Then we selected the records with known parental forms of hybrids and searched for their taxonomic status, geographic localization and genetic identity (including accession numbers of their cytochrome *b* gene sequences when available, see below). These records included both crosses between species and crosses between different subspecies or races. We did not distinguish reciprocal crosses (i.e., with no respect to which of the hybridizing species is maternal and which is paternal; such data are scarce) in further analyses.

To qualify as a hybridizable cross, at least one of the

hybrid offspring must have been hatched alive (if data were available) or this was inferred from the presence of viable later-generation hybrids. For each individual cross, we recorded the fertility of F₁ hybrid. The presence of viable backcrosses, F₂ and later-generation hybrids suggest a potential for the gene flow (Table 2). We distinguished the hybridization records based on the occurrence of bisexual homoploid hybrids from those based on obligatorily parthenogenetic species. We also noted whether the cross originated from the wild or captivity, the mechanisms of sex determination of the species (temperature or genetic), estimation of the divergence time by TimeTree (Hedges et al., 2006) and genetic distance between the parental species. For the purpose of the genetic distance estimation, we downloaded the cytochrome b gene sequences of parental species from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>), with the exception of some species, for which cytochrome b gene has not been sequenced yet. In such cases, we used the phylogenetically closest sister species that had their cytochrome b gene sequenced. In the family Lacertidae, *Podarcis raffonei* was substituted for *P. tiliguerta*, *P. wagleriana* was substituted for *P. filfolensis*, *Darevskia alpina* was substituted for *D. saxicola* and in the family Phrynosomatidae, *Phrynosoma goodei* was substituted for *P. platyrhinos*. Nevertheless, there were no equally possible substitutions for some parental species, and thus, we downloaded other available mitochondrial genes: 12 S and 16 S mtDNA for *Aspidoscelis burti*, *A. inornata*, *A. gularis*, *A. sexlineata*, *A. tigris*, *Cnemidophorus lemniscatus*, *C. gramivagus* (Teiidae), *Gymnophthalmus cryptus*, *G. speciosus* (Gymnophthalmidae), *Leiolepis belliana*, *L. guttata*, *L. reevesii* (Agamidae), *Sphaerodactylus nicholsi*, *S. townsendi* (Sphaerodactylidae), *Woodworthia maculata* (Gekkonidae), only 12S mtDNA for *Phrynosoma coronatum*, *P. blainvillii*, *P. cerroense* (Phrynosomatidae) and for three lineages of *Podarcis hispanicus* (Lacertidae), *NADH2* gene for *Heteronotia binoi* SM6, *H. binoi* CA6 (Gekkonidae), *Phrynosoma wigginsi*, *P. cerroense* (Phrynosomatidae), *NADH4* gene for *Iguana iguana*, *I. delicatissima*, *Ctenosaura pectinata*, *C. hemilopha* (Iguanidae), *Lampropholis coggeri* (Scincidae) and *NADH1* gene for *Sceloporus cowlesi* and *S. tristichus* (Phrynosomatidae). For *Sceloporus undulatus*, *S. woodi* and the chromosomal races of *S. grammicus* (Phrynosomatidae), only these parts of mtDNA longer than 2,000 base pairs were available: cytochrome oxidase subunit 3, tRNA-Gly, presumptive protein 3, tRNA-Arg, presumptive protein 4L, presumptive protein 4,

tRNA-His, tRNA-Ser, tRNA-Leu (see Supplementary Materials). The cytochrome b gene was chosen by virtue of having sequences available in GenBank for the largest range of hybridizable lizard species and a faster mutation rate.

The sequences were aligned using BioEdit version 7.0.5.3 (Hall, 1999) and the alignments were manually optimized. For each parental species pair, the alignment had different length from 282 bp to 2,429 bp. Genetic distances between the species (see Table 2) were calculated using uncorrected p distance (that is frequently used in similar studies, e.g., Lijtmaer et al., 2003; Meganathan et al., 2010) and the HKY 85 model, with the transition-transversion ratio estimated from the data in PAUP* version 4.0b10 (Swofford 2002). We selected HKY 85 model as a reliable compromise between the number of parameters and precision (see Salemi et al., 2009), the model parametrizing nucleotide frequencies and transition transversion ratio has been shown to be appropriate for cyt b data in related species across the vertebrate taxa (e.g., Kotlík et al., 2006; Tang et al., 2006).

We collected data about 93 hybridization events in which the parental species were identified. One additional report of between-generic mating *Ctenosaura* x *Iguana* demonstrates failure of precopulatory RIMs, but only in the combination of these parental species. For four species pairs of geckos, no molecular data were available (see Table 2). Finally, we collected data about 73 bisexual hybrids and 16 unisexual parthenogenetic species. Nevertheless, through inspection of the literature, we found eight records of hybridizations (6 from captivity, 2 from nature) suggesting that the attempts to reproduce the hybrids were not sufficient, e.g., a low number of F₁ hybrids without further breeding attempts. Finally, we statistically analyzed the homogenous set of 65 bisexual species and separately the set of 16 parthenogenetic species.

In the following analyses, we adjusted the genetic distances calculated from 12 S, 16 S, *NADH2* and *NADH4* to cytochrome b genetic distances. With respect to the different mutation rate of the individual genes (Eo and DeWoody, 2010), we estimated the rate coefficients for the above mentioned genes to cytochrome b. We calculated their ratios on the basis of the mean distance calculations for 9 pairs of 12 S and 16 S genes, 12 pairs of 12 S genes independently, 9 pairs of *NADH2* genes and 7 pairs of *NADH4* genes and also the mean of cytochrome b gene distances for the same pairs in separate groups. Using these ratios, we counted the theoretical

Table 2 Reported cases of hybridization in lizards and selected vertebrate groups

Parental species	Snout-vent length (mm)		Gene flow	Nature/Captivity	Partheno-gensis	Sex determination	Genetic distance of parental species		Sequence length (bp)	Divergence time (my)	References
	1	2					HKY85	uncorrected P			
Agamidae											
<i>Phrynocephalus puijattai</i>	84 ^{#1}	77	-?	N		ZW	0.051	0.049	288	3.8*	1
<i>Leiolepis belliana</i>	156 ^{#2}	151 ^{#3}	+	N		?	0.027 ^a	0.027 ^b	493		2
<i>Leiolepis guttata</i>	184 ^{#3}	151 ^{#3}	B	N	P	?	0.169 ^a	0.147 ^b	493		2-4
Iguanidae sensu lato											
<i>Amblyrhynchus cristatus</i>	548 ^{#4}	480 ^{#4}	+	N		XY ¹	0.158	0.140	923		5, 6
<i>Ctenosaura pectinata</i> Colima	353	353	+	N		XY ¹	0.039 ^s	0.037 ^h	561		7
<i>Ctenosaura pectinata</i> Colima	353	353	+	N		XY ¹	0.078 ^s	0.072 ^h	561		7
<i>Ctenosaura pectinata</i>	353	400	+	N		XY ¹	0.113 ^s	0.102 ^h	561		8
<i>Ctenosaura bakeri</i>	315	510	+	N		XY ¹	0.135	0.122	1036	5.8***	9-12
<i>Iguana iguana</i>	380	580	+	N		XY ¹	0.155 ^s	0.135 ^h	903		13
<i>Iguana iguana</i>	380	412	M	C		XY ¹					14
<i>Ctenosaura bakeri</i> X <i>C. similis</i>	57 ^{#5}	59 ^{#5}	+	N/C		XY ¹	0.027	0.026	306		15
<i>Anolis osa</i>	55	51	+	N		XY	0.150	0.132	957	3 - 6.4 *****	16
<i>Anolis krugi</i>	74	80	+	N		XY	0.183	0.158	988		17
<i>Anolis trinitatis</i>	146	125	+	N		XY ¹	0.025	0.024	416		18-20
<i>Gambelia wislizenii</i>	131	137	+	N		XY	0.037	0.035	423		20, 21
<i>Crotaphytus collaris</i>	131	95 ^{#6}	+	N		XY	0.115	0.104	423		20, 22, 23
<i>Crotaphytus collaris</i>	55	70	+	N		XY ¹	0.130	0.117	724		24, 25
<i>Liolaemus gracilis</i>	87 ^{#7}	87 ^{#7}	+	N		XY ¹	0.045 ⁱ	0.043 ^j	969		26
<i>Sceloporus tristichus</i> South	91	65	+	N		XY	0.091 ⁱ	0.084 ^j	2016	1.0*	27
<i>Sceloporus undulatus undulatus</i>	81	81	-	N		XY	0.070 ⁱ	0.066 ^j	2429		28
<i>Sceloporus grammicus</i> F6	81	81	+	N		XY	0.100 ⁱ	0.091 ^j	2429		29-32
<i>Sceloporus grammicus</i> F5	81	81	-	N		XY	0.112 ⁱ	0.102 ^j	2429		33
<i>Sceloporus grammicus</i> FM2	80 ^{#8}	87 ^{#7}	+	N		XY ¹	0.116 ⁱ	0.105 ^j	969		26
<i>Sceloporus grammicus</i> FM2	81	81	+	N		XY	0.122 ⁱ	0.111 ^j	2429		28, 33
<i>Sceloporus cowlesi</i>	81	81	-	N		XY	0.143 ⁱ	0.127 ^j	2429		28
<i>Sceloporus grammicus</i> F6	81	81	+	N		XY					
<i>Sceloporus grammicus</i> FM3	81	81	-	N		XY					

Continued Table 2

Parental species	Snout-vent length (mm)		Gene flow	Nature/Captivity	Partheno-gensis	Sex determination	Genetic distance of parental species		Sequence length (bp)	Divergence time (my)	References
	1	2					HKY85	uncorrected P			
<i>Phrynosoma wigginsi</i>	79	85	+	N		XY ¹	0.012 ^e	0.012 ^f	1033		34
<i>Phrynosoma coronatum</i>	100	85	-?	C		XY ¹	0.054 ^e	0.052 ^d	765		35
<i>Phrynosoma blainvillii</i>	100	114	-?	C		XY ¹	0.055 ^e	0.053 ^d	754		35
<i>Phrynosoma mcallii</i>	83 ^{#9}	88 ^{#9}	+	N		XY ¹	0.138	0.122	687		36, 37
<i>Phrynosoma coronatum</i>	114	130	+	C		XY ¹	0.185	0.159	1044		35
Gekkonidae sensu lato											
<i>Sphaerodactylus nicholsi</i>	25	28	+	N		?	0.004 ^a	0.004 ^b	883	6,0*	38
<i>Heteronotia binoei</i> SM6	55	55	+	N	P	ZW	0.15 ^e	0.131 ^f	1284		39
<i>Nactus pelagicus</i>	57 ^{#10}	63 ^{#4}	-	N	P	?	0.068	0.064	378	> 2,0**	40
<i>Gekko tawaensis</i>	71	74 ^{#3}	+	N		XY ²					41
<i>Gekko yakuensis</i>	72	80	+	N		ZW ²					42
<i>Phyllodactylus guttatus</i>	87 ^{#11}	78 ^{#1}	-	N/C		?					43
<i>Phyllodactylus tuberculatus</i>	72 ^{#12}	81	-	N		ZW ²					44
<i>Woodworthia maculata</i> Little	57 ^{#13}	67 ^{#13}	+	N		?	0.035 ^a	0.032 ^b	600		45
Teiidae											
<i>Aspidoscelis gularis</i>	115 ^{#14}	64 ^{#15}	B	N	P	GSD ²	0.124 ^a	0.110 ^b	771		46, 47
<i>Cnemidophorus lemniscatus</i>	93 ^{#16}	69 ^{#17}	B	N	P	GSD ²	0.127 ^a	0.112 ^b	763		48
<i>Aspidoscelis burri</i>	140	85	no B	N	P	GSD	0.140 ^a	0.123 ^b	787		49
<i>Aspidoscelis tigris</i>	100 ^{#18}	115 ^{#14}	B	N	P	XY	0.168 ^a	0.146 ^b	774		50
<i>Aspidoscelis deppoi</i>	87 ^{#19}	107 ^{#20}	B	N	P	GSD ²	0.172	0.151	671		51, 52
<i>Aspidoscelis tigris</i>	100 ^{#18}	64 ^{#15}	B	N	P	XY	0.195 ^a	0.167 ^b	791		51, 53, 54
<i>Aspidoscelis tigris</i>	100 ^{#18}	85	B	N	P	XY	0.213 ^a	0.181 ^b	792		50, 55, 56
<i>Kentropyx striata</i>	127 ^{#21}	119 ^{#22}	no B	N	P	GSD ²	0.153	0.134	819		57
<i>Tupinambis merianae</i>	500	614	+	N		?	0.078	0.073	300		58
Gymnophthalmidae											
<i>Gymnophthalmus cryptus</i>	28	45	no B	N	P	XY	0.141 ^a	0.125 ^b	1764		59

Continued Table 2

Parental species	Snout-vent length (mm)		Gene flow	Nature/ Captivity	Partheno - genesis	Sex determination	Genetic distance of parental species		Sequence length (bp)	Divergence time (my)	References
	1	2					HKY85	uncorrected P			
Lacertidae											
<i>Darevskia caucasica</i>	64 ^{#23}		+	N		ZW	0.087	0.080	1045		60, 61
<i>Darevskia mixta</i>	63		+	N		ZW	0.103	0.094	1045		60, 61
<i>Darevskia mixta</i>	63		+	N		ZW	0.125	0.112	582		60, 61
<i>Darevskia rudis</i>	85 ^{#23}		+	N		ZW	0.131	0.116	1045		60, 61
<i>Darevskia alpina</i>	65		+	N		ZW	0.142	0.125	582		60, 61
<i>Darevskia caucasica</i>	64 ^{#23}		+	N		ZW	0.142	0.125	582		60, 61
<i>Darevskia mixta</i>	63		B	N	P	ZW	0.143	0.125	1045		60-64
<i>Darevskia mixta</i>	63		B	N	P	ZW	0.150	0.131	1045		61, 64
<i>Darevskia derjugini</i>	65		+	N		ZW	0.164	0.143	1045		60, 61
<i>Darevskia narensis</i>	76 ^{#23}		B	N	P	ZW	0.167	0.144	1044		61-64
<i>Darevskia parvula</i>	57		+	N		ZW	0.170	0.145	1045		60, 61
<i>Darevskia alpina</i>	65		+	N		ZW	0.179	0.153	582		60, 61
<i>Darevskia saxicola</i>	70 ^{#23}		+	N		ZW	0.179	0.153	582		60, 61
<i>Darevskia raddei</i>	76		B	N	P	ZW	0.182	0.155	1044		61, 64, 65
<i>Iberolacerta monticola</i>	85		+	N		ZW	0.045	0.043	598		66
<i>Lacerta schreiberi</i> EAST	135		+	N		ZW	0.042	0.040	816		67
<i>Lacerta viridis</i>	150		+	C		ZW	0.060	0.057	403	3,3*	68, 69
<i>Lacerta viridis</i>	150		-?	C		ZW	0.135	0.121	306		70
<i>Lacerta viridis viridis</i>	150		-?	C		ZW	0.148	0.131	388		68
<i>Lacerta schreiberi</i>	135		+	C		ZW	0.159	0.141	306	8,6*	68, 69
<i>Lacerta strigata</i>	112 ^{#23}		-?	C		ZW	0.164	0.144	306		68
<i>Lacerta trilineata</i>	153		+	C		ZW	0.166	0.144	403		69, 71
<i>Lacerta viridis</i>	150		+	C		ZW	0.172	0.148	405		72
<i>Lacerta viridis</i>	150		+	C		ZW	0.176	0.151	405		68, 69, 73
<i>Lacerta viridis meridionalis</i>	150		-	C		ZW	0.178	0.153	405		68
<i>Lacerta pamphylica</i>	120		-?	C		ZW	0.179	0.153	405		68
<i>Lacerta schreiberi</i>	135		+	C		ZW	0.191	0.163	663		68, 69, 73
<i>Podarcis muralis</i> Tuscan	80		+	N		ZW	0.031	0.030	887		74, 75

Continued Table 2

Parental species	Snout-vent length (mm)		Gene flow	Nature/Captivity	Parthenogenesis	Sex determination	Genetic distance of parental species		Sequence length (bp)	Divergence time (my)	References
	1	2					HKY85	uncorrected <i>P</i>			
<i>Podarcis muralis</i> East France	80	80	+	N		ZW	0.055	0.052	887		74, 75
<i>Podarcis bocagei</i>	70	74	+	N		ZW	0.098	0.089	282		76
<i>Podarcis hispanicus</i> Valencia	74	74	+	N		ZW	0.117	0.111	379		77
<i>Podarcis hispanicus</i> Valencia	74	74	+	N		ZW	0.137	0.128	379		77
<i>Podarcis bocagei</i>	70	64 ^{#1}	+	N/C		ZW	0.156	0.135	288		78, 79
<i>Podarcis sicula</i>	90	85	-	N		ZW	0.156	0.137	831		80
<i>Podarcis sicula</i>	90	87	-?	N		ZW	0.161	0.141	674		81
<i>Podarcis sicula</i>	90	76	+	N		ZW	0.171	0.148	859		80
<i>Podarcis sicula</i>	90	74	+	N		ZW	0.182	0.156	887	12.2*	82
<i>Timon lepidus lepidus</i>	260	260	+	N		ZW	0.131	0.116	627		83, 84
<i>Timon lepidus</i>	260	170	-	C		ZW	0.142	0.126	627		85
<i>Zootoca vivipara</i> South France	71 ^{#23}	71 ^{#23}	+	N		ZW	0.014	0.014	427		86
<i>Zootoca vivipara vivipara</i>	71 ^{#23}	71 ^{#23}	+	C		ZW	0.040	0.039	1143		87
<i>Zootoca vivipara vivipara</i>	71 ^{#23}	71 ^{#23}	+	N		ZW	0.058	0.055	1143		88
Scincidae											
<i>Carlia rubrigularis</i> North	60	60	+	N		?	0.154	0.135	399		89
<i>Lampropholis coggeri</i> North	45	45	+	N		XY	0.174 ^h	0.152 ^h	836		90
<i>Oligosoma otagense</i>	133	125	+	N		GSD	0.090	0.083	613		91
<i>Plestiodon latiscutatus</i>	80 ^{#23}	90 ^{#1}	+	N		GSD	0.126	0.113	910		92
Fishes											
<i>Leptisosteus osseus</i>			-	C		GSD	0.145	0.127	1141	33.1 * - 100 ****	93
<i>Acantharchus pomotis</i>			-	C		GSD	0.202	0.174	1140	33.6*	94
<i>Pomoxis nigromaculatus</i>			-	C		GSD	0.221	0.187	1140	28.9*	94
Frogs											
<i>Pseudacris regilla</i>			-	N		GSD	0.167	0.147	597		95
<i>Pseudacris crucifer</i>			-	N		GSD	0.214	0.181	623	22.1*	95
Snakes											
<i>Pituophis catenifer sayi</i>			+	N		ZW	0.119	0.107	1109	27.9*	96
Turtles											
<i>Chelonia mydas</i>			-	N		TSD ³	0.109	0.098	429	62.8*	97

Continued Table 2

Parental species	Snout-vent length (mm)		Gene flow	Nature/Captivity	Partheno-genesis	Sex determination	Genetic distance of parental species		Sequence length (bp)	Divergence time (my)	References
	1	2					HKY85	uncorrected P			
<i>Mauremys reevesii</i>			-	C		TSD ³	0.132	0.118	1140		98
<i>Mauremys sinensis</i>			-	C		TSD ³	0.142	0.125	1036		99
<i>Cuora flavomarginata</i>			?	N/C		TSD ³	0.169	0.146	1140		100
Crocodyles											
<i>Crocodylus rhombifer</i>			+	C		TSD ²	0.127	0.113	1156		101
Birds											
<i>Anas platyrhynchos</i>			-	C		ZW ²	0.141	0.125	1033		28,9*
Mammals											
<i>Balaenoptera bonaerensis</i>			+	C		XY ²	0.055	0.052	383		7,6*
<i>Tursiops truncatus</i>			-	C		XY ²	0.093	0.085	1086		7,0*
<i>Tursiops truncatus</i>			-	C		XY ²	0.097	0.088	1086		8,9*

“Gene flow” denotes cases with at least partial fertility of hybrids and production of backcrosses by F₁ hybrids or parthenogens of hybrid origin (potential gene flow). Gene flow is coded as present (“+”); it means presence of backcrosses or higher order hybrids; “-”: absence of later hybrids is uncertain, “B”: it means presence of backcrosses in parthenogens, “noB”: it means obligatory parthenogens, “M”: denotes between-species mating without production of hybrids. TSD = temperature sex determination; XY and ZW refers to different modes of GSD. Divergence time was computed by TimeTree (Hedges et al. 2006, <http://www.timetree.net>) or was added from literature.

Snout-vent length: Meiri (2008); #1 Uetz and Hošek (2014); #2 Manthey and Grossmann (1997); #3 Chirio and LeBreton (2007); #4 Zug (2013); #5 Köhler et al. 2010; #6 Axtell (1972); #7 Leache and Cole (2007); #8 Bateman and Chung-MacCoubrey (2012); #9 Young (2010); #10 Wilson and Swan (2010); #11 El Din (2006); #12 Köhler (2000); #13 Fitness et al. (2012); #14 Ramírez-Bautista et al. (2009); #15 Pérez-Ramos et al. (2010); #16 Montgomery et al. (2011); #17 Mesquita and Colli (2003); #18 Walker (1981); #19 Mata-Silva and Ramirez-Bautista (2005); #20 Beargie and McCoy (1964); #21 Gallagher and Dixon (1992); #22 Vitt (1991); #23 Bannikov et al. (1977).

Sex determination: Pokorná and Kratochvíl (2009); ¹ Rovatsos et al. (2014); ² Gamble (2010); ³ Janzen and Phillips (2006).

Genetic distance: ^a estimated from 12S and 16S rRNA gene divergences using rate coefficient 2.0374; ^b estimated from 12S and 16S rRNA gene divergences using rate coefficient 1.9014; ^c estimated from 12S rRNA gene divergences using rate coefficient 2.1104; ^d estimated from 12S rRNA gene divergences using rate coefficient 2.1000; ^e estimated from NADH2 gene divergences using rate coefficient 1.1555; ^f estimated from NADH2 gene divergences using rate coefficient 1.1374; ^g estimated from NADH4 gene divergences using rate coefficient 1.2401; ^h estimated from NADH4 gene divergences using rate coefficient 1.219; ⁱ substituted by distances computed from composite sequences of mitochondrial genes.

Divergence time: * Hedges et al. (2006); ** Moritz (1987); *** Gutsche and Köhler (2008); **** Herrington et al. (2008); ***** Brandley and de Queiroz (2004).

References of hybridization: 1. Jin and Liu (2008); 2. Grismer et al. (2014); 3. Malysheva et al. (2006); 4. Grismer and Grismer (2010); 5. Rassmann et al. (1997); 6. Lückler and Feiler (2002); 7. Zarza et al. (2011); 8. Zarza et al. (2008); 9. Köhler and Blinn (2000); 10. Gutsche and Köhler (2004); 11. Gutsche and Köhler (2008); 12. Pasachnik et al. (2009); 13. Breuil (2000); 14. Dirksen (2004); 15. Köhler et al. (2012); 16. Jezkova et al. (2013); 17. Gorman et al. (1971); 18. Montanucci (1970); 19. Montanucci (1978); 20. McGuire et al. (2007); 21. Montanucci (1974); 22. Axtell (1972); 23. Montanucci (1983); 24. Morando et al. (2007); 25. Olave et al. (2011); 26. Leache and Cole (2007); 27. Jackson (1973); 28. Arévalo et al. (1993); 29. Reed and Sites (1995); 30. Reed et al. (1995a); 31. Reed et al. (1995b); 32. Sites et al. (1995); 33. Hall and Selander (1973); 34. Montanucci (2004); 35. Baur (1984); 36. Leache and McGuire (2006); 37. Mulcahy et al. (2006); 38. Murphy et al. (1984); 39. Moritz (1983); 40. Eckstut et al. (2013); 41. Toda et al. (2006); 42. Toda et al. (2001); 43. Werner and Sivan (1996); 44. Dixon (1996); 45. Fitness et al. (2012); 46. Abuheba (2001); 47. Walker et al. (1989); 48. Cole and Dessauer (1993); 49. Moritz et al. (1989b); 50. Dessauer et al. (1996); 51. Taylor et al. (2001); 52. Hernandez-Gallegos et al. (2003); 53. Parker and Selander (1976); 54. Taylor et al. (2000); 55. Dessauer et al. (2000); 56. Manning et al. (2005); 57. Cole et al. (1995); 58. Cabana et al. (2014); 59. Cole et al. (1993); 60. reviewed by Murphy et al. (2000); 62. Arakelyan (2002); 63. Danielyan et al. (2008); 64. Kupriyanova (2009); 65. MacCulloch et al. (1997); 66. Arribas et al. (2014); 67. Godinho et al. (2008); 68. Rykena (1991); 69. Rykena (2002); 70. Rykena and Henke (1978); 71. Peek (1978); 72. Mayer and Tiedemann (1985); 73. Rykena (1996); 74. Schulte et al. (2013); 75. Schulte et al. (2013); 76. Pinho et al. (2007); 77. Renoult et al. (2009); 78. Galán (2002); 79. Pinho et al. (2009); 80. Capula (1993); 81. Capula (2002); 82. Gorman et al. (1975); 83. Nunes et al. (2011); 84. Miraldo et al. (2013); 85. Bischoff (1981); 86. Mila et al. (2013); 87. Arrayago et al. (1996); 88. Lindtke et al. (2010); 89. Phillips et al. (2004); 90. Singhal and Moritz (2012); 91. Chapple et al. (2012); 92. Okamoto et al. (2013); 93. Herrington et al. (2008); 94. Bolnick and Near (2005); 95. Wilson et al. (1974); 96. LeClere et al. (2012); 97. Karl et al. (1995); 98. Buskirk et al. (2005); 99. Schilde et al. (2004); 100. Otani (1995a, b) ex Buskirk et al. (2005); 101. Polet et al. (2002); 102. Prager and Wilson (1975); 103. Glover et al. (2013); 104. Caballero and Baker (2010); 105. Zhang et al. (2014).

values of cytochrome *b* gene distances for the parental species pairs with unknown sequences of this gene.

In order to determine which factors, if any, predict the fertility of F_1 hybrids and thus the possibility for a gene flow, we used marginal models (GEE - Generalized Estimating Equation approach; it is GLM class model enabling correction for intra-class correlations among observations). The fertility of the F_1 hybrids and thus the potential for a gene flow was given as a dependent variable with binomial distribution. The genetic distance of hybridizable pairs was given as a continuous predictor. Bisexual/pathenogenetic reproduction mode of the hybrids and natural versus artificial origin of the crossing were both introduced as categorical explanatory variables. The identity of the principal clades present in our data set (i.e., Gekkota, Iguania, Lacertidae, Teiidae/Gymnophthalmidae and Scincidae) was included in the model to account for phylogenetic dependence of the species data. The calculations were performed using `geeglm` function of `geepack` package in the R environment (R Core Team, 2013).

For each successfully hybridizing species pair, we calculated a ratio between the snout-vent length of the smaller parental species and that of the larger one (Fig. 3). These ratios, expressed in percents, were further referred to as a similarity in body sizes of the parental species. The ratio was set to 100% for within-species hybridizations in which the relevant body sizes were not available for both parental subspecies.

Visualisation of the hybridization events on a tree depicting phylogenetic relationships among families was done using `Mesquite` package (Maddison and Maddison, 2009). For a reconstruction of ancestral states of the hybridization presence/absence, we chose the maximum parsimony method. The topology of the tree was adopted from Pyron et al. (2013).

2 Results

2.1 List of hybridization events among genetically distinct lizard species/subspecies

We gathered literature records describing the hybridization in 94 pairs of genetically distinct lizard species/subspecies; 78 of which produced bisexual hybrids (61 and 17 from wild and captivity, respectively) while the remaining 16 pairs were parental forms that gave rise to parthenogenetic species. The families represented the most often were the Lacertidae (42 pairs), Phrynosomatidae (13 pairs), Teiidae (9 pairs), Iguanidae (7 pairs), Gekkonidae (5 pairs), Scincidae (4 pairs), Crotophytidae (3 pairs), Dactyloidae (3 pairs), Agamidae (3

pairs), and Phyllodactylidae (2 pairs); Sphaerodactylidae, Liolaemidae and Gymnophthalmidae were each represented by a single species pair. Altogether, 13 of 42 families of extant lizards (Squamata without snakes) were represented in this list and their distribution on the phylogenetic tree (cf. Pyron et al., 2013) suggests that the hybridization events can be found in multiple clades across the tree topology (see Fig. 1). The distribution of the hybridizing species pairs among the principal clades of the lizards is, however, highly biased in favour of the most studied clades; the hybridizing species belong to the Lacertoidea (52 pairs), Iguania (30 pairs), Gekkota (8 pairs) and Scincoidea (4 pairs), (see Table 2).

In almost all cases, the hybridizing pair belongs to the same genus. The only exception is the hybridization between the two morphologically and ecologically distinct, but phylogenetically closely related species of the Galapagos iguanas *Amblyrhynchus cristatus* and *Conolophus subcristatus*. However, their hybrids are viable and at least partially fertile (Rassmann et al., 1997; Lückner and Feiler, 2002).

2.2 Genetic divergence between parental forms of viable bisexual hybrids and parthenogenetic hybrid species

In our dataset (Table 2), the mean genetic distances within pairs of parental species computed from the mitochondrial DNA sequences (cyt *b* gene, HKY85 model) were higher in the parthenogenetic hybrid species (0.154, $n = 16$) than in the viable bisexual hybrids (0.113, $n = 73$). Non-parametric Mann-Whitney test revealed that this difference is statistically significant ($Z = 2.69$, $P = 0.0071$). The lowest genetic distance between the parental forms of the parthenogenetic hybrid species was 0.068 in *Nactus pelagicus* and *N. multicarinatus* (Gekkonidae, Eckstut et al., 2013), while 21 of the 73 distances computed for the parental pairs of bisexual hybrids were smaller to this value (the lowest value was 0.004 for *Sphaerodactylus nicholsi* and *S. townsendi*; Sphaerodactylidae, Murphy et al., 1984). In contrast to this, the maximum value (0.213) for the parental species pair of the parthenogenetic hybrid species (*Aspidoscelis tigris* and *A. inornata*; Teiidae, Dessauer et al., 1996) was close to that found in the parents of the bisexual hybrids (0.191, *Lacerta agilis* and *L. schreiberi*, Lacertidae, Rykena, 2002; see Figure 2).

2.3 Genetic distance of parental species and a potential for gene flow

Most of the parthenogenetic hybrid species (12 of 16) were reported to produce viable hybrids with at least one of their parental species. Even the *Aspidoscelis neo-*

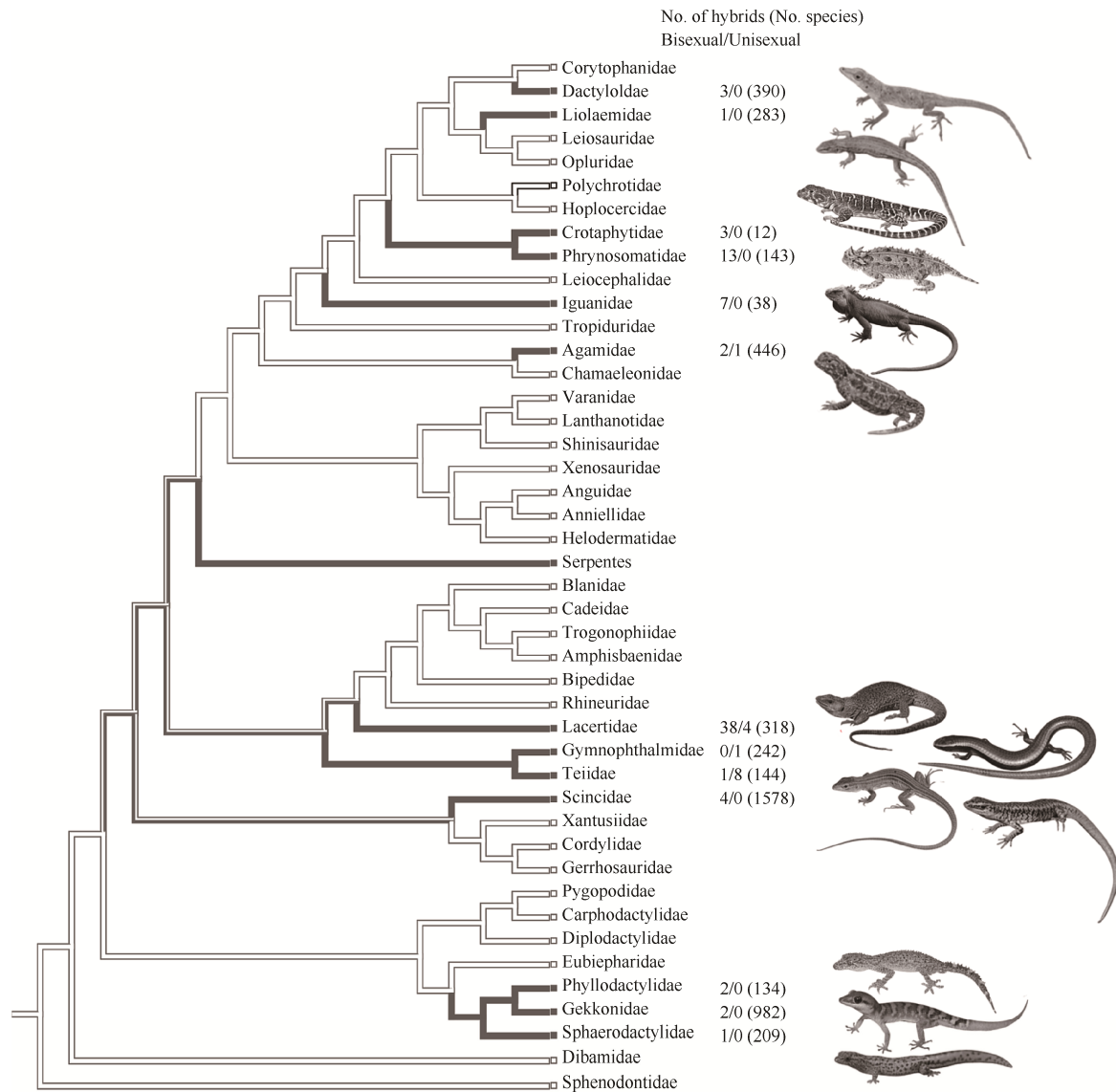


Fig. 1 Families of extant lizards which are represented in this list and their distribution on the phylogenetic tree (cf. Pyron et al., 2013) suggests that hybridization events can be found in multiple clades across tree topology

The distribution of hybridizing species pairs among the principal clades of lizards is, however, highly biased; the hybridizing species belong to Lacertoidea (52), Iguania (30), Gekkota (8 pairs) and Scincoidea (4 pair), see Table 2.

mexicana, a unisexual parthenogenetic hybrid of the bisexual species *A. tigris* and *A. inornata*, is still able to backcross with both of the parental taxa (Teiidae, Des-sauer et al., 1996; 2000; Manning et al., 2005, for details see Table 2). Fertility of such hybrids is often prevented by the parthenogenetic mode of the reproduction itself and/or polyploidy (Dowling and Secor, 1997). These specific mechanisms have been repeatedly reviewed (Fujita and Moritz, 2009) and thus, we further focused only on the bisexual diploid hybrids.

In 59 of 73 parental pairs of bisexual hybrids, a potential gene flow (for definition see under the Materials and Methods) has been reported. Thus, the potential for

a gene flow was not proved in only 14 pairs of the parental species! Moreover, clear evidence against such a gene flow was available in just six of these pairs. The marginal geeglm of our dataset (Table 2) accounting for the phylogenetic clade revealed that the genetic distance between the parental species has no effect on the presence/absence of the potential gene flow ($\chi^2 = 0.60$, $P = 0.4369$). This result has remained unchanged ($\chi^2 = 0.59$, $P = 0.4424$) when eight uncertain cases (six of which coming from breeding experiments in captivity) were excluded. No effects of captivity/wild origin of the data as well as relative difference between the parental species in their body sizes were found.

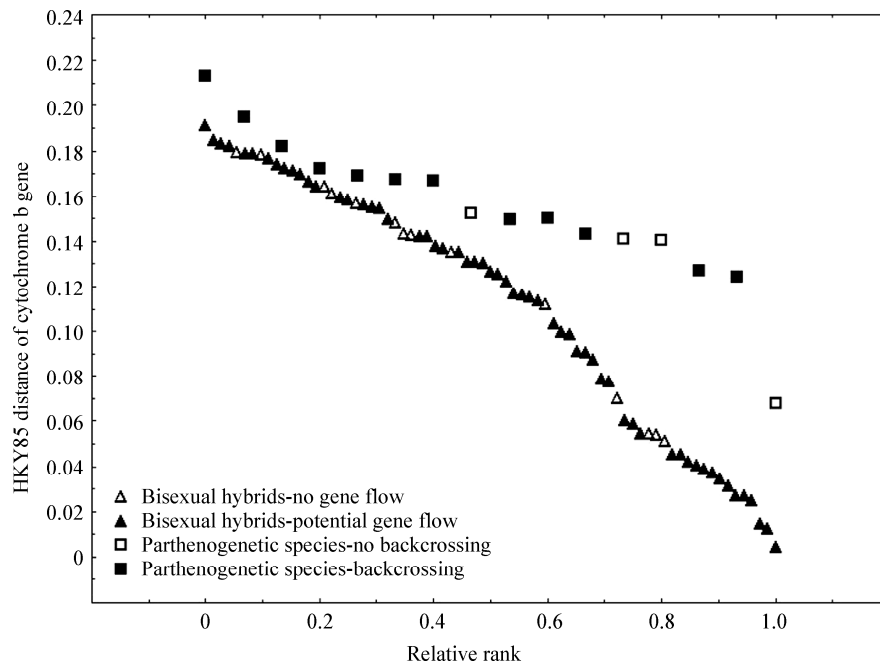


Fig. 2 Plot of genetic divergences between hybridizing parental species, given as HKY85 distance in nucleotide sequence of mitochondrial *cyt b* gene, against relative rank order of this value

Ranks of bisexual homoploid hybrids (triangles) and parthenogens of hybrid origin (squares) were treated separately. Cases with at least partial fertility of hybrids and production of backcrosses were documented close to upper limits of divergence in both F_1 hybrids and hybrid parthenogens. These cases are denoted by filled marks (triangles and squares).

The most genetically distant parental species of the bisexual hybrids have documented a potential for a gene flow. The viable and fertile F_1 hybrids were experimentally proved in *Lacerta agilis* and *L. schreiberi* exhibiting genetic distance of 19% (Lacertidae, Rykena, 2002), *Phrynosoma coronatum* and *P. cornutum* (18%; Phrynosomatidae, Baur, 1984). Natural hybridization between parents with greater divergence was also reported. Hybridization between *Anolis trinitatis* and *A. aeneus* (18%; Dactyloidae) showed that the reproductive function was affected and thus the backcross hybrids were rare in nature (Gorman et al., 1971). Nevertheless, the fertile hybrids of the species pairs exhibiting comparable genetic distances were also repeatedly detected in nature: e.g., *Podarcis sicula* and *P. melisellensis* (18%), *P. sicula* and *P. wagleriana* (17%; Gorman et al., 1975; Capula, 1993), *Darevikia saxicola* and *D. brauneri* (18%; Lacertidae; MacCulloch et al., 1997; for review see Fu, 1999; Murphy et al., 2000).

2.4 Body size differences within pairs of hybridizing species

The minimum value of the similarity in body sizes of the parental species was 56% in the case of a parthenogenetic hybrid of the *Aspidoscelis gularis* and *A. sexlineata* (Teiidae). The median value was 92% and only 10 percent of the values were smaller than 72% (Fig. 3).

The similarity in body sizes of the parental species was higher in the bisexual hybrids (median = 93%, percentile 10 = 75%, minimum = 62%) than in the parthenogenetic hybrid species (median = 84%, percentile 10 = 61%; Mann-Whitney test: $Z = -2.80$, $P = 0.0050$) and this difference has remained significant even when the hybrids of the genetically related species ($\text{HKY85} < 0.068$, i.e., that between *Nactus pelagicus* and *N. multicarinatus*) were excluded ($n_{\text{bisexual}} = 53$, $n_{\text{parthenogenetic}} = 16$, $Z = -2.28$, $P = 0.0225$).

3 Discussion

3.1 List of hybridizing species/subspecies

The number of reliable literature records of hybridization between distinct species of lizards is surprisingly small, especially when compared with the huge number of such records available in other vertebrates, in particular birds, mammals, turtles, and fishes (Grant and Grant, 1992; Galgon and Fritz, 2002; Fitzpatrick, 2004; Bolnick and Near, 2005; Buskirk et al., 2005; McCarthy, 2006). The hybridization records are heavily biased towards taxa occurring in Europe (lacertids) and North America (iguanaids, phrynosomatids and teiids), where lizard faunas are relatively poor, but herpetological research has the longest tradition. Thus, it is likely that some hybrids of lizard species may have been over-

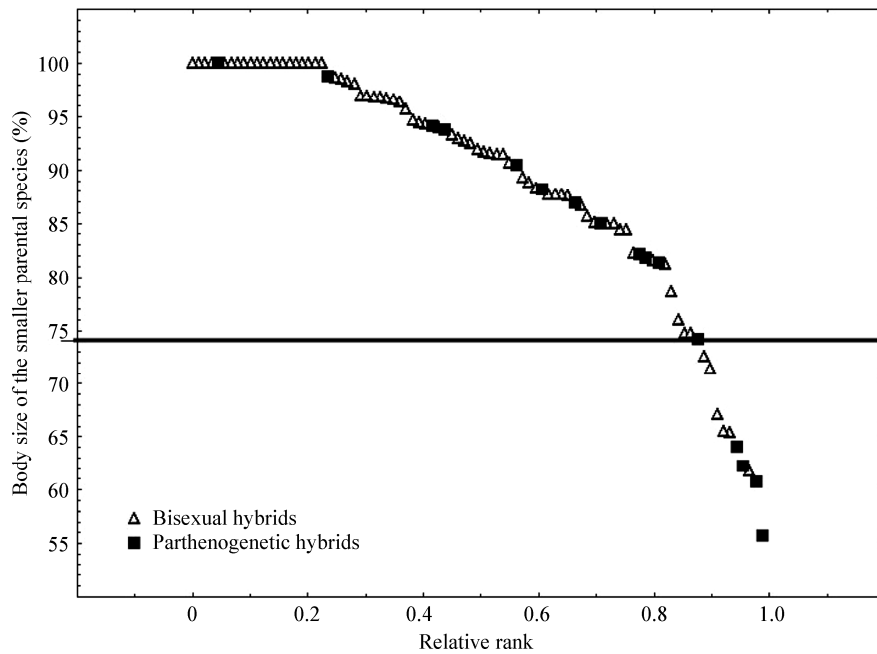


Fig. 3 Body size differences within pairs of hybridizing species, i.e., the ratio between snout-vent length of the smaller and larger-bodied species (in percents), plotted against the relative rank of this value in our sample (data are ordered from the smallest value to the maximum and scaled to the total number of the examined parental pairs)

The line shows the value 0.74, which was previously reported for Squamata by Shine and Charnov (1992) as mean relative ratio between the body size at sexual maturity and the maximum adult body size.

looked by scientists rather than entirely absent. This view is further supported by putative hybrid records between lizard and (especially) snake species, which are occasionally reported by hobbyists (Hoser, 1988; 1991; Lásková, 2006). However, these reports were usually too poorly documented to be included in our analyses.

It is surprising that our list is missing hybridization examples of species with temperature-determined sex. It is known that many phylogenetically divergent species of turtles and crocodiles with temperature-determined sex often hybridize (Conceicao et al., 1990; Karl et al., 1995; Harding and Davis, 1999; Parham et al., 2001; Fritz and Mendau, 2002; Galgon and Fritz, 2002; Ray et al., 2004; Schilde et al., 2004; Buskirk et al., 2005; Rodriguez et al., 2008; Weaver et al., 2008). One could assume that species without differentiated sex chromosomes would hybridize more successfully than species with genetically determined sex, in which a higher degree of sterility frequently occurs in the heterogametic sex with XY or ZW chromosomes (Haldane, 1922; Presgraves, 2010). Unfortunately, we were unable to verify this hypothesis in our study.

3.2 Genetic divergence between parental forms of viable bisexual hybrids and parthenogenetic hybrid species

Moritz et al. (1989a) predict that there should be a

threshold of divergence between bisexual species below which hybrids do not reproduce parthenogenetically. In agreement to this, the parental species of parthenogens in our sample are typically genetically well-differentiated species (> 0.123 sequence divergence, except the case of *Nactus* 0.068). Our review also revealed that the upper limit of the genetic distances between parental species is approximately the same in both parthenogenetic (0.213 for *Aspidoscelis tigris* x *A. inornata*) and bisexual (0.191 for *Lacerta schreiberi* x *L. agilis*) hybrids (see Table 2). In contrast to the parthenogens, the divergence between the parental forms of bisexual hybrids covers a full range, including the zone of close similarity (e.g., Toda et al., 2001; 2006).

3.3 Genetic divergence between parental species/subspecies and potential for gene flow

The absence of a significant relationship between the genetic distance of the parental species and the potential for a gene flow (the presence of fertility in F_1 hybrids and viable later hybrids) was surprising as this relationship was previously demonstrated in various animal taxa (cf. Edmands, 2002; Sánchez-Guillén et al., 2014; but see Lessios and Cunningham, 1990). In lizards, the most complex hybridization experiments were carried out in a series of species belonging to the genus *Lacerta* sensu stricto (Rykena and Henke, 1978; Rykena, 1991;

1996; 2002). These studies reported that an increasing phylogenetic distance of the hybridizing species was positively associated with an increasing proportion of sterile hybrids, especially in females. Genetic introgression was enabled by crossing fertile males with the parental species (Rykena, 2002). In our dataset, we did not find a significant relationship between the genetic distance of the parental species and the potential for a gene flow, probably due to the statistical distribution of the data. The vast majority of F_1 hybrids usually appeared fertile in lizards, allowing the existence of backcrosses with at least one parental species. These results may be affected due to lack of variance in presence/absence coded data. Moreover, reliable records of hybridization among lizard species are scarce and possibly affected by a publication bias against negative results. Thus, we cannot exclude the existence of more genetically divergent species pairs, which are able to produce infertile hybrids but still have remained unexplored. Alternatively, the hybridization of genetically more divergent species of lizards is constrained by the divergence of sexual and/or species recognition signals and consequent emergence and completion of precopulatory isolation mechanisms prior to the appearance of entirely infertile hybrids (cf. Price, 1998; Servedio, 2001; Coyne and Orr, 2004 but see Gage et al., 2002).

One would expect that in a laboratory, where are no ecological differences keeping the lizards apart, a greater success in hybridization could be achieved. However, we did not find any evidence of distantly related species in captivity hybridizing at a more successful rate than those in nature. It may be argued, however, that in the wild, infertile F_1 hybrids may be easily recognized, but due to their infrequent origin, they may be easily overlooked.

3.4 Hybridization, taxonomy and conservation

A high occurrence of hybrid fertility and thus at least a theoretical chance for an introgression of some genes from species to species has serious potential consequences for understanding of lizard diversity. Despite the increasing genetic divergence in lizards, the potential for hybridization may further complicate the application of the biological species concept, which postulates interbreeding of natural populations that are reproductively isolated from other such groups (Mayr, 1942). Moreover, taxonomic recommendation regarding the status of reptilian subspecies are biased towards splitting; when the genetic analyses (genetic distance values) are used, then subspecies are more likely elevated to the status of a species without regard to any

species concept (Torstrom et al., 2014).

These findings suggest that artificial hybridization occurring in nature (mainly in secondary contact zone) is common. We must be cautious and do not underestimate the situation, especially when it concerns small populations of endangered species of lizards. Relatively good fertility of hybrids leading to gene introgression could result in the merging of species and the extinction of the endangered species (Allendorf et al., 2001; Rhymer and Simberloff, 1996). Where possible, it is good to control and limit the introduction of non-native species, while establishing a genetically pure population in captivity, which would be able to reproduce and, in the future, allow for the reintroduction to the areas where the species has already gone extinct (e.g., in *Iguana Breuil*, 2000). However, where populations are very small and vulnerable to other factors (such as domestic animal introduction, destruction of natural habitats, etc.), then it is not effective to discriminate lizards to small taxonomic units on the basis of only genetic differences and thus protect too small population of lizards. Moreover on the basis of empirical studies, the inbreeding depression threat of small population is more urgent than the potential disadvantages of outbreeding (Edmands, 2007). When protecting a species, we need to approach the problem of its survival individually, building a plan tailored to the particular species. It is because hybridization can have quite different consequences in individual cases (Allendorf et al., 2001). When a protection management plan of an endangered species is discussed, not only the genetic distance between both hybridizing species needs to be considered, but also the context of the environment and selection pressures.

3.5 Body size differences within pairs of hybridizing species

Differences in body size may contribute not only to pre-mating isolation mechanisms, but also to postzygotic RIMs (Bolnick et al., 2006). We found that body size differences within pairs of parental species reported to hybridize are typically small. In 90% of bisexual hybrids, the body size of smaller parental species represented more than 75% of the body size of the larger one. This value is close to the 74% reported for a typical relation of the body size at maturity to the maximum body size reported within lizard species (around 70% for other reptiles; Shine and Charnov, 1992; Shine and Iverson, 1995). Thus, the body size differences between the hybridizing lizard species are comparable to those among conspecifics participating in reproduction.

Although the statistical distribution of lizard body sizes was carefully examined (Meiri, 2008), reliable estimates of this distribution in species pairs having opportunities to hybridize in nature have remained unexplored. It is due to non-trivial interactions between phylogenies, character displacement and biogeography. Thus, it is still impossible to directly test the deviations of the observed body size differences between the hybridizing species pairs from the expected distribution of this variable.

3.6 Comparison of lizards with other principal clades of vertebrates

It seems that lizards are similar to fishes and frogs in the ability to produce hybrids when genetic distances measured as the sequence divergence of *cyt b* gene between the parental species approach 21%, but this genetic distance is higher than that found in hybrids of snakes, turtles, crocodylians, birds, and mammals, which belong to the record holders in hybridization between a lot of phylogenetically distant species (Table 2).

The rates of sequence divergence in mitochondrial genes reported between parental species of lizards are considerably higher than those reported in other clades of vertebrates (e.g., mammals, turtles, crocodiles, birds, but not snakes see Nabholz, 2009; Eo and DeWoody, 2010 and references herein). Also, the nuclear genes of squamates exhibit a faster evolutionary rate than birds, turtles and crocodiles (Hughes and Mouchiroud, 2001). Thus, high values of the genetic divergence between parental species of lizard hybrids do not mean longer elapsed time from the last common ancestor of the hybridizing species. Nevertheless, a supposed constant ratio between evolutionary rates of mitochondrial and nuclear genes (but see Grechko, 2013 for criticism of widespread misuse of the mitochondrial genes) would still suggest that lizards are able to hybridize with less similar genomes than other vertebrates. The data presented here support the idea that a gene flow may exist between congeneric lizard species, and are consistent with the general idea of the semipermeable nature of species boundaries given by Flegr (2013) and Harrison and Larson (2014). The idea of continuity between varieties and species has been proposed by Charles Darwin (Darwin, 1859). Recently, this issue was addressed again by Mallet (2008a, b). The divergence of hybridizing species can be maintained despite the gene flow, due to varying permeability of particular genome region, therefore the hybridizing taxa often remain distinct for only a part of the genome (Harrison and Larson, 2014).

An important question, which our review could not

address, is whether particular divergences between the parental species are associated with either beneficial (hybrid vigour) or detrimental effects (outbreeding depression, genetic incompatibility, etc.) on fitness in lizards. Most of the available records reporting between-species hybrids come either from field studies relying on molecular evidence but lacking fitness parameters, or from casual observations made by private breeders. Properly documented experimental hybridizations are surprisingly rare. The absence of such evidence calls for further experimental studies.

In conclusion, we found that lizards are exceptional among vertebrates in their ability to hybridize despite being highly genetically divergent. Reliable records of hybridization are scarce, however, probably due to an insufficient effort devoted to this topic. We also found that despite high genetic divergence (roughly up to 20% of mitochondrial *cyt b* gene sequences), the hybridizing species are usually morphologically similar enough to be formally classified as congeners by current taxonomists. Lastly, our review revealed that more data on the occurrence of hybridization in lizards are necessary, both for better understanding of the role of hybridization in evolution and for better planning in conservation efforts, an aspect that has remained unexplored. Our review has revealed much with regard to the limits of successful lizard hybridization. To further explore these limits, we must gather more experimental evidence of hybridization between distant lizard species, including pairs of species more divergent than those known to produce fertile hybrids.

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Author Contributions Conceived the idea: DF, EL, JLL. Analyzed the data: DF, JLL. Wrote the paper: JLL, DF, EL.

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Supplementary Materials: Accession numbers of the mitochondrial gene sequences of the parental species downloaded from the Genebank

Species	Cyt <i>b</i>	12S	16S	NADH2	NADH4	mtDNA
Agamidae						
<i>Leiolepis belliana</i>			AF378379			
<i>Leiolepis guttata</i>			AF378377			
<i>Leiolepis reevesii</i>			AF378376			
<i>Phrynocephalus putjata</i>	KF691634					
<i>Phrynocephalus vlangalli</i>	KF691642					
Iguanidae sensu lato						
<i>Amblyrhynchus cristatus</i>	AY948118				U66234	
<i>Conolophus subcristatus</i>	AY948122				U66235	
<i>Ctenosaura bakeri</i>	GU331976				EU407507	
<i>Ctenosaura hemilopha</i>					U66227	
<i>Ctenosaura pectinata</i> Colima					EU246700	
<i>Ctenosaura pectinata</i> Balsas					EU246769	
<i>Ctenosaura pectinata</i> North					EU246713	
<i>Ctenosaura pectinata</i>					EU246730	
<i>Ctenosaura similis</i>	GU331975				EU407509	
<i>Iguana delicatissima</i>					AF217783	
<i>Iguana iguana</i>					AF217786	
<i>Anolis aeneus</i>	EU557103			AF055950		
<i>Anolis krugi</i>	GU057654					
<i>Anolis osa</i>	HQ641730					
<i>Anolis polylepis</i>	HQ641741					
<i>Anolis pulchellus</i>	GU057619					
<i>Anolis trinitatis</i>	AF493592			AY909781		
<i>Gambelia sila</i>	EU037370			EU038401		
<i>Gambelia wislizenii</i>	EU037415			EU038446		
<i>Crotaphytus bicinctores</i>	EU037682			EU038711		
<i>Crotaphytus collaris</i>	EU037482			EU038513		
<i>Crotaphytus reticulatus</i>	EU037745			EU038774		
<i>Liolaemus bibroni</i>	JN410531					
<i>Liolaemus gracilis</i>	JN410538					
<i>Sceloporus cowlesi</i>						EF031648
<i>Sceloporus grammicus</i> F5						L32581
<i>Sceloporus grammicus</i> F6						L32580
<i>Sceloporus grammicus</i> FM2						L32585
<i>Sceloporus grammicus</i> FM3						L32583
<i>Sceloporus grammicus</i> HS						L32579
<i>Sceloporus grammicus</i> LS						L32578
<i>Sceloporus tristichus</i> North						EF031668
<i>Sceloporus tristichus</i> South						EF031890
<i>Sceloporus tristichus</i> West						EF031657
<i>Sceloporus undulatus undulatus</i>						AF440075

Continued Table

<i>Species</i>	<i>Cyt b</i>	12S	16S	NADH2	NADH4	mtDNA
<i>Sceloporus woodi</i>						AF440089
<i>Phrynosoma blainvillii</i>		GQ279564				
<i>Phrynosoma cerroense</i>		GQ279507		DQ385347		
<i>Phrynosoma cornutum</i>	AY141087	DQ385390		DQ385344		
<i>Phrynosoma coronatum</i>	AY141097	DQ385396		DQ385349		
<i>Phrynosoma goodei (platyrhinos)</i>	(EU543746)	DQ385391		DQ385345		
<i>Phrynosoma mcallii</i>	AY141098	DQ385402		DQ385355		
<i>Phrynosoma wigginsi</i>				DQ385348		
Gekkonidae <i>sensu lato</i>						
<i>Sphaerodactylus nicholsi</i>		KC840509	KC840603			
<i>Sphaerodactylus townsendi</i>		KC840513	KC840607			
<i>Heteronotia binoi</i> CA6				DQ000967		
<i>Heteronotia binoi</i> SM6				DQ000789		
<i>Nactus multicaudatus</i>	KC581486			JQ627854		
<i>Nactus pelagicus</i>	KC581545			JQ627855		
<i>Woodworthia maculata</i> Large			HM542435			
<i>Woodworthia maculata</i> Little			HQ343302			
Teiidae						
<i>Aspidoscelis angusticeps</i>	KF555516				KF555554	
<i>Aspidoscelis burti</i>		AY046428	AY046470			
<i>Aspidoscelis deppiei</i>	AF006303	AY046431			KF555559	
<i>Aspidoscelis gularis</i>		AY046443	AY046485			
<i>Aspidoscelis inornata</i>		AY046436	AY046478			
<i>Aspidoscelis sexlineata</i>		AY046445	AY046487			
<i>Aspidoscelis tigris</i>		AY046452	AY046494			
<i>Cnemidophorus gramivagus</i>		AY046432	AY046474			
<i>Cnemidophorus lemniscatus</i>		AY046438	AY046480			
<i>Kentropyx calcarata</i>	JQ639739	AY046458	AY046500			
<i>Kentropyx striata</i>	JQ639672	AY046460	AY046502			
<i>Tupinambis merianae</i>	KF034084					
<i>Tupinambis rufescens</i>	KF034091					
Gymnophthalmidae						
<i>Gymnophthalmus cryptus</i>			AF101362			
<i>Gymnophthalmus speciosus</i>			AF101368			
Lacertidae						
<i>Darevskia alpina (saxicola)</i>	(U88617)					
<i>Darevskia brauneri</i>	AF206181					
<i>Darevskia caucasica</i>	U88616					
<i>Darevskia clarkorum</i>	U88605					
<i>Darevskia daghestanica</i>	AF206171					
<i>Darevskia derjugini</i>	AF206172					
<i>Darevskia mixta</i>	AF147796					
<i>Darevskia nairensis</i>	AF164081					

Continued Table

<i>Species</i>	<i>Cyt b</i>	12S	16S	NADH2	NADH4	mtDNA
<i>Darevskia parvula</i>	U88609					
<i>Darevskia portschinskii</i>	U88615					
<i>Darevskia raddei</i>	AF164076					
<i>Darevskia rudis</i>	U88614					
<i>Darevskia saxicola</i>	U88617					
<i>Darevskia valentini</i>	U88611					
<i>Iberolacerta galani</i>	HQ234901					
<i>Iberolacerta monticola</i>	HQ234897					
<i>Lacerta agilis</i>	AF373032	AF149947	DQ494823		NC021766	
<i>Lacerta bilineata</i>	AF233415	AF149957	AY714979			
<i>Lacerta media israelica</i>	KC896975	KC896891	KC896947			
<i>Lacerta pamphylica</i>	DQ097089					
<i>Lacerta schreiberi</i>	AF372103	EF422436	DQ097097			
<i>Lacerta schreiberi</i> EAST	AF386785					
<i>Lacerta schreiberi</i> WEST	AF386784					
<i>Lacerta strigata</i>	DQ097091	DQ097095	DQ097099			
<i>Lacerta trilineata</i>	AF233427	AF149953	AF149969			
<i>Lacerta viridis</i>	AF233425	AF149962	KC621334		KC621628	
<i>Lacerta viridis meridionalis</i>	AM087228					
<i>Podarcis bocagei</i>	AF372087	AF469421			EF081132	
<i>Podarcis carbonelli</i>	AF372079	AF469418			EF081152	
<i>Podarcis hispanicus</i>	AF372084	AF469443			DQ081163	
<i>Podarcis hispanicus</i> Valencia		HQ898210				
<i>Podarcis hispanicus hispanicus</i>		HQ898179				
<i>Podarcis hispanicus liolepis</i>		HQ898166				
<i>Podarcis melisellensis</i>	AY185036	AY185004				
<i>Podarcis muralis</i> East France	DQ001029					
<i>Podarcis muralis</i> Tuscany	DQ001028					
<i>Podarcis muralis</i> Venetian	HQ652905					
<i>Podarcis raffonei</i> (tiliguerta)	(JX852113)	AJ250157			KJ027980	
<i>Podarcis sicula</i>	AY770890	AY770907		EU006727	KF372035	
<i>Podarcis tiliguerta</i>	JX852113	DQ017658		JX852139		
<i>Podarcis wagleriana</i> (filfolensis)	(KF022066)	DQ017659		(KF022078)	KJ027979	
<i>Timon lepidus lepidus</i>	JX626302			DQ902256	DQ902324	
<i>Timon lepidus nevadensis</i>	JX626247					
<i>Timon pater</i>	AF378964			DQ902258	DQ902326	
<i>Zootoca vivipara carniolica</i>	AY714929	AF247375	AF247050			
<i>Zootoca vivipara louislantzii</i>	AY714919	AF247372	AF247047			
<i>Zootoca vivipara vivipara</i>	AY714913	AF247370	AF247045			
<i>Zootoca vivipara</i> North Spain	AF247998					
<i>Zootoca vivipara</i> South France	AF248003					
Scincidae						
<i>Carlia rubrigularis</i> North	AF181042					

Continued Table

<i>Species</i>	<i>Cyt b</i>	12S	16S	NADH2	NADH4	mtDNA
<i>Carlia rubrigularis</i> South	AF181056					
<i>Lampropholis coggeri</i> North					HM029922	
<i>Lampropholis coggeri</i> South					HM029999	
<i>Oligosoma otagense</i>	JN999970			JN999934		
<i>Oligosoma waimatense</i>	JN999978			JN999942		
<i>Plestiodon japonicus</i>	EU203134					
<i>Plestiodon latiscutatus</i>	EU203035					
Fishes						
<i>Atractosteus spatula</i>	JF912043					
<i>Lepisosteus osseus</i>	JF912059					
<i>Acantharchus pomotis</i>	AY115994					
<i>Micropterus salmoides</i>	AY115999					
<i>Pomoxis nigromaculatus</i>	AY115992					
Frogs						
<i>Pseudacris crucifer</i>	AY210883					
<i>Pseudacris nigrita</i>	KJ536229					
<i>Pseudacris regilla</i>	KJ536196					
<i>Pseudacris triseriata</i>	KJ536224					
Snakes						
<i>Pantherophis vulpinus</i>	FJ267681					
<i>Pituophis catenifer sayi</i>	AF337112					
Turtles						
<i>Caretta caretta</i>	AY678314					
<i>Chelonia mydas</i>	EU918368					
<i>Cuora flavomarginata</i>	AY434606					
<i>Cyclemys shanensis</i>	AJ604513					
<i>Geoemyda japonica</i>	AY434602					
<i>Mauremys reevesii</i>	AY434567					
<i>Maremys sinensis</i>	AY434615					
<i>Sacalia quadriocellata</i>	AY434618					
Crocodiles						
<i>Crocodylus rhombifer</i>	HQ595019					
<i>Crocodylus siamensis</i>	GU331906					
Birds						
<i>Anas platyrhynchos</i>	EU585609					
<i>Anser anser</i>	EU585613					
Mammals						
<i>Balaneoptera acutorostrata</i>	HM034299					
<i>Balaenoptera bonaerensis</i>	HM034297					
<i>Grampus griseus</i>	AF084059					
<i>Sotalia guianensis</i>	DQ086827					
<i>Tursiops truncatus</i>	JN571480					

II.

Divergence in sexual behaviour during distant and close hybridization in eublepharid geckos: experimental crossing of *Eublepharis macularius* and its congeners

Eva Landová, Jitka Jančúchová-Lásková, Lukáš Kratochvíl, Jakub Polák, Daniel

Frynta

(manuscript)

**Divergence in sexual behaviour during distant and close hybridization in
eublepharid geckos: experimental crossing of *Eublepharis macularius* and its two
congeners**

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Abstract

In allopatry, precopulatory reproductive isolation mechanisms are not directly favoured by selection, instead are a side-effect of genetic differentiation driven by selection on other traits. In sympatric or parapatric situations reinforcement may cause high divergence in sexual behaviour, even in genetically similar lineages. In our study, we experimentally crossed geckos at two levels: 1) two morphologically distinct, genetically distant species, *E. macularius* and *E. angramainyu*, living in allopatry and 2) closely related but morphologically and genetically distinct “dark” and “yellow” forms of *E. macularius* (family Eublepharidae). Despite more than 12-15 million years of isolation between these two species, differences in sexual behaviour do not lead to a complete isolation as hybridization frequently occurs. We found quantitative differences during interspecific hybridization, such as higher frequency of tongue flicking in females and later male ejaculation when mating with a heterospecific female that took longer to allow copulation. Interspecific differences in female aggressive behaviour towards males during hybridization gave more advantages to bigger *E. angramainyu* males. Although the pattern of sexual behaviour of hybrids is more like that of *E. angramainyu*, postzygotic RIMs prevent successful reproduction of the hybrids with this parental species, while it is possible with the second parental species, *E. macularius*. These differences in sexual behaviour may serve as precopulation reproduction isolation mechanisms in the potential future contact. Furthermore, we found asymmetry in fitness cost during hybridization between the yellow and dark form. The former one had lower incubation success when hybridizing with the dark form, whilst the dark form hatchability was the same in either intraspecific mating or hybridization. Despite clear differences in female sexual behaviour between the yellow and dark form during hybridization, these have not yet served as a precopulation isolation mechanism. The cost

of distant hybridization is higher compared to the close one, but the fitness costs and benefits are apparently asymmetric. The yellow form is more permissive for both the distant and close interspecific hybridization, but only the former one may be advantageous for its offspring. These asymmetric gains during hybridization are frequent in nature even if the species are able recognise an allospecific and heterospecific partner. The level of isolation depends not only on genetic, morphological and behavioural differences, but also on ecological conditions selecting for future differentiation of reproductive isolation between species/lineage.

Keywords. Hybridization, Introgression, Precopulatory barriers, Epigamic behaviour, Lizards, *Eublepharis angramainyu*

Introduction

Hybridization is an important evolutionary process that can cause diversification and adaptation (Schluter 2001, Seehausen 2004; Arnold 1992; Abbott et al. 2013, Hedrick 2013). This may give rise to new species, but these may also disappear through fusion (Rhymer & Simberloff 1996, Wolf et al. 2001, Perry et al. 2002, Olden et al. 2004, Todesco et al. 2016). Reproductive isolating mechanisms (RIMs) prevent emergence of hybrids and merging of sexually reproducing species. The prezygotic RIMs cause either a premating isolation or postmating gametic incompatibilities (e.g., fecundity may be lowered due to incompatibilities between sperm proteins and egg receptors, decreased viability of sperm etc.; examples are reviewed in Servedio 2001). Different preferences of sexual partners and/or other mating behavioural patterns play an important role in the premating isolation between the species. If these prezygotic barriers are not fully developed and copulation occurs, the gene introgression between species can prevent postzygotic RIMs such as inviability or sterility of the hybrids (Haldane 1922; Orr & Presgraves 2000; Coyne & Or 2004). These disadvantages put pressure on individuals to be able to recognize a heterospecific species and avoid it. Similarly, there is a strong pressure to create premating barriers preventing the emergence of hybrids. The other reproductive isolating mechanisms like some postmating, prezygotic incompatibilities or postzygotic barriers as reduced hybrid fertility following heterospecific mating are connected with substantial fitness lost for parental species (Servedio 2001). These premating isolating mechanisms are clearly pivotal in maintaining reproductive barriers in sympatric species/lineages (Heatcote et al. 2016) and after a secondary contact of two distinct previously allopatric species (Servedio 2001).

On the other hand, it appears that hybridization is quite common in nature and can significantly contribute to speciation of both plants and animals (Mallet 2007, Genovart

2009, Abbott et al. 2013). The ability to hybridize and produce viable or even fertile hybrids varies significantly across major vertebrate clades (reviewed by Jančúchová-Lásková et al. 2015a). It is known that mammals lost this ability earlier than fish, birds, or turtles (Wilson et al. 1974, Prager & Wilson 1975, Karl et al. 1995, Fitzpatrick 2004, Bolnick & Near 2005). In our review, we found that lizards are special in their ability to hybridize and produce fertile hybrids despite being highly genetically divergent (Jančúchová-Lásková et al. 2015a).

With increasing genetic divergence of two species/lineages, their phenotypic divergence may also grow, which can facilitate speciation by reducing the likelihood to hybridize due to a different sexual partner choice (Heatcote et al. 2016). The less the potential hybrids are viable and fertile, the more pressure is exerted on the ability to recognize the conspecific sexual partner. It is assumed that it is more important for females to choose a partner correctly because their investment in offspring is usually higher (Wirtz 1999, Randler 2002, Heatcote et al. 2016). However, during the initial stages of secondary contact the male discrimination may play a more important role in limiting hybridization due to a lack of historical selection (Echelle & Connor 1989, Rhymer & Simberloff 1996, Huxel 1999).

When individuals of one species engage in reproductive activities with mates of another species as happens during hybridization, and when these interactions reduce the fitness of one or both species, we call this sexual or reproductive interference (Gröning & Hochkirch 2008). Many studies have suggested that costly interspecific sexual interactions (i.e., reproductive interference) such as interspecific mating can also explain exclusive patterns among closely related species (reviewed by Gröning & Hochkirch 2008, Kyogoku 2015). Despite a selection pressure against costly interspecific mating in geckos, there is evidence of interspecific sexual

interference that is also the main mechanism of displacement between examined species (*Hemidactylus frenatus* and *Hemidactylus garnotii*). Male *H. frenatus* courted and copulated with both conspecific and heterospecific females and showed a preference for larger *H. garnotii* females (Dame & Petren 2006). Understanding the differences in costs of sexual behaviour that promote or erode an isolation between species/lineages, especially those involving parental species and hybrids, has broader implications for evolutionary processes like reinforcement, ecological speciation, or extinction of novel or parental genetic lineages (for review see Schluter 2001).

Although there is a limited number of studies directly showing loss of fitness during hybridization in reptiles (Jančúchová-Lásková et al. 2015a), much more attention was devoted to proximate causes of sexual and reproductive behaviour (reviewed in Crews et al. 1998, Rhen & Crews 2002, Kratochvíl et al. 2008, Schořálková et al. 2017). Among reptiles, the leopard geckos (*Eublepharis macularius*), a species with temperature sex determination (TSD, Viets et al. 1993), was used as a model for behavioural and physiological experiments demonstrating interplay between temperature and steroid hormones that both have organizational and activation effects on adult sexual behaviour (Rhen & Crews 2000). Adult females manipulated with high levels of testosterone for a long period of time were less attractive and nonreceptive for males and more aggressive. Medium levels of testosterone had an opposite effect on female receptivity (Rhen et al. 1999). A similar effect on female sexual behaviour has incubation of eggs in male biased incubation temperature (Flores & Crews 1995). Leopard geckos have perceptual and cognitive abilities to recognize chemical signals of the same and opposite sex (Mason & Gutzke 1990, Steele & Cooper 1997) as well as familiar and novel individuals (LaDage & Ferkin 2006, 2007). In geckos and other reptiles, there are chemo-signals used in intraspecific communication that can have reproductive

consequences (for review see Martin & Lopez 2014). However, the ability to discriminate against hybrids based species-specific chemicals or differences in sexual behaviour by pure species/lineages have not been studied in this species. Leopard geckos can hybridize with distinct allopatric species *E. angramainyu* and this hybridization produces F1 hybrids that are viable and fertile. The introgression of *E. angramainyu* genes into the *E. macularius* genome can be enabled via backcrossing (Jančúchová-Lásková et al. 2015b). Hybridization between closely related, but genetically and morphologically distant lineages (forms) may occur as well (preliminary data, Lásková 2008, master theses).

In our study, we experimentally crossed geckos at two levels – 1) two distinct and genetically distant species, *E. macularius* and *E. angramainyu*, and 2) “yellow” and “dark” forms belonging to the *E. macularius* species complex (family Eublepharidae). These represent more closely related forms than the former dyad.

E. macularius (Blyth, 1854) is a common laboratory animal widely used as a model species of squamate reptiles. Its distribution range includes large territories of Afghanistan, Pakistan, and India (Seufer et al. 2005). Our “yellow” form of *E. macularius* genetically and morphologically corresponds to the previously described “yellow” form, while our “dark” form corresponds to the *E. cf. fuscus* species according to Starostová et al. (2005) and Kratochvíl & Frynta (2002), who used the same stock our tested animals are descendant from. Based on the data reported by Starostová et al. (2005), there was 2.8% of sequential divergence in 12S and 16S mtDNA between these “yellow” and “dark” forms.

E. angramainyu is, on the other hand, less common, inhabiting Mesopotamia and SW Iran (Anderson 1999). Its range is separated from that of *E. macularius* by the Iranian Plateau and Zagros Mountains (Seufer et al. 2005). Sequence divergence between

mitochondrial genes of the *E. macularius* and *E. angramainyu* are considerable (uncorrected p-distances for 303 bp fragment of cyt b gene exceed 19%; HKY85 distance 22%, Palupčíková unpublished data) and fully congruent with the geological dates of the main uplift of the Iranian Plateau. Both geological and genetic evidence suggests that a divergence of the two species happened at least 12-15 million years ago (details in Jančúchová-Lásková et al. 2015b). As we have already proven, this species can hybridize and produce viable offspring (see Jančúchová-Lásková et al. 2015b), which is an example of hybridization among very distant parental species inside the squamate reptiles (Jančúchová-Lásková et al. 2015a).

Long allopatric isolation may be the mechanism responsible for potential between species differences (Schluter 2001, Funk 2006, Rundle 2013), e.g. in sexual behaviour during conspecific mating or differences during hybridization experiments (McKinnon et al. 2004, Vines & Schluter 2006). Even though these hybridization interactions produce viable hybrids, their next reproduction success is limited. In our study, focused on experimental interspecific hybridization between *E. macularius* and *E. angramainyu*, the hybrids have the best reproductive success during mating with one of the species (backcross) - *E. macularius*. However, hybrid mating with other hybrids or the second parental species, *E. angramainyu*, was not successful (see Jančúchová-Lásková 2015b). Thus, the hybrid's sexual behaviour is important too. In the case of future secondary contact of this species, hybrid's mating pattern similarity with *E. macularius* and consequently preference for such mating partner would be a clear advantage. Theoretically, our experimental system of interspecific hybridization is a good model for studying how differences in sexual behaviour correspond to fitness costs between these two species and their hybrids, which may be important if these two species (or similarly distant species of Squamata) came into contact in the future.

The sexual interference may influence the reproductive success of two forms-species of *E. macularius* as well. In our preliminary data, intraspecific hybridization between these two forms led to reduced egg hatchability (Lásková 2008, master theses) and this disadvantage might have caused their increased differences in sexual behaviour, which helped increase reproductive isolation barriers between the “yellow” and “dark” forms. Testing of this hypothesis requires not only a precise measurement of differences in sexual behaviour in both sexes during hybridization, but also an expression of fitness gains for hybrids, parentals, and backcrosses via surviving eggs and juveniles.

These two levels of hybridization among different forms (subspecies) and very distant interspecific hybridization between *E. macularius* and *E. angramainyu* provide a unique opportunity to study: (1) between-species differences in courting and mating behaviour and (2) compare them with possible raising differences in precopulation RIM between two distinct forms of *E. macularius* species complex. Our aim is to test several predictions about possible differences in sexual behaviour of *E. macularius* and *E. angramainyu*. For interspecific hybridization (1), regarding to 12-15 million years of separation between the two species, we predict: (a) clear between-species differences in sexual behaviour (courting as well as mating behaviour should be affected), (b) clear distinction of sexual behaviour during hybridization from sexual behaviour during intraspecific mating attempts of both parental species, (c) similar behaviour of the hybrid to the *E. macularius* behavioural pattern to promote advantage of producing viable backcrosses; (2) slight differences in sexual behaviour between two forms of *E. macularius* species complex. As females of this species invest more energy into reproduction, we predict (3) bigger differences in female sexual hybridization during interspecific hybridization as well as (4) during hybridization between forms. Due to the fact, that males and females inevitably interfere, we predict that (5) specific male

courting behaviours involve the female's decision to accept the specific male (female receptivity) more than the others and we try to assess the key parameters of male behaviour involving female receptivity.

Materials and Methods

Ethical Statement

All the performed experiments were approved by the institutional Animal Care and Use Committee of Charles University in Prague and the Ethical Committee of Ministry of Education, Youth and Sports of the Czech Republic license no. 18147/203 and 24773/2008 - 10001. After the study, the geckos were used either for other experiments or for breeding purposes.

Experimental animals and housing

For our mating experiments, we used 68 females (56 "yellow" form, 9 "dark" form) and 24 (15 "yellow" form, 9 "dark" form) males of the leopard gecko, *E. macularius*, 18 females and 19 males of a rare species, *E. angramainyu*, and 9 females and 3 males of hybrids between these species. All animals were in adult age (at least two years old). Eight out of 60 individuals of *E. macularius* and 13 of 37 of *E. angramainyu* were imported from Pakistan or Iran, respectively (for more details see Jančúchová-Lásková et al. 2015b). Other animals were the first generation of descendants of these wild-caught animals. The hybrids originated from mating females *E. macularius* and a male *E. angramainyu*. All individuals of parental species/forms were sexually experienced and had reproduced successfully in previous breeding seasons (for more details see Jančúchová-Lásková et al. 2015b and Starostová et al. 2005). The number of mating interactions is provided in Supplements 1 and 2.

The animals of the “yellow” form correspond fully to those described as a “yellow” and “dark” form to the *Eublepharis* cf. *fuscus* (Börner, 1981) species in Starostová et al. (2005), because they come from the same imports and laboratory stocks. However, determination according the morphological traits is not fully reliable in *E. fuscus* (Seufer 2005) and genetic characterization of geographically localized individuals are not available now, for that reason we use a more precise term “dark” form. These forms differ genetically (Starostová et al. 2005) as well as morphologically in their coloration, body size, and shape (see Lásková 2008, master theses). In our breeding records, the mean and maximal value of snout-vent length (SVL) are 130.3 mm and 145.4 mm (n = 51) in the “yellow” form, and 104.5 mm and 119.1 mm in the “dark” form, respectively. The second species, *E. angramainyu* (Anderson and Leviton, 1966), is the largest eyelid gecko of Eublepharidae family. The mean SVL is 154.0 mm and maximum is 165.6 mm (n = 12). All the measured animals were older than two years. These mean body size measures correspond to the means of SVL reported in Starostová (2005). *E. macularius* and *E. angramainyu* species differ in the coloration pattern and body shape too (Jančúchová-Lásková et al. 2015b).

All the animals were kept individually in glass cages (60 x 30 x 20cm or 30 x 30 x 20cm in size) with a bark substrate, shelter, water dish, and a dish for mealworms. The temperature in the breeding room was about 28°C. The geckos had continuous access to water and were fed by crickets and mealworms dusted with vitamins and minerals (Nutri Mix) weekly; AD₃ and E vitamins were provided once per 14 days.

Methods of testing

The mating experiments were carried out in a temperature-controlled breeding room (28°C) after 7 p.m. with respect to the geckos’ nocturnal activity. Prior to the experiment, the females were weighed and checked for their receptivity by a visual

inspection of the follicular growth through the abdomen wall (Rhen et al. 2000, see below for more details). Next, we gently placed the male into the female's terrarium for 30 min and recorded copulation behaviour using a night vision video camera. The terrarium was illuminated by a single red 25-W light bulb. If mating did not occur within this interval, we repeated the trial another day. Each experimental female was allowed to copulate exclusively with a single male during a given mating season, because the geckos of the genus *Eublepharis* are able to store sperm for several months (Kratochvíl & Frynta 2002, LaDage et al. 2008). In contrast, males were allowed to copulate with multiple females within a single breeding season. The experiments were performed in seasons 2005 – 2009, 2013 and 2015 (lasting from January/February to May/June).

Courting and mating behaviour in E. macularius and E. angramainyu during conspecific and heterospecific mating

Male sexual behaviour

Males react to the female presence by tongue flicking, which is behaviour linked to vomeroolfaction in geckos and other reptiles (reviewed in Mason & Parker 2010). Tongue flicks were directed in our experiments to scent marks on the substrate or directly to various parts of the female body. Sex recognition cues seem to be related to skin lipids in the leopard gecko, some fatty acids are shared by both sexes. However, several steroid analogues of cholesterol are unique to males while long-chain methyl ketones are unique to females (Mason & Gutzke 1990). These chemicals are used as sex pheromones and males respond by more frequent tongue flicking and other courting behaviours to the presence of unfamiliar female semiochemicals (Steele & Cooper 1997). Males of *E. macularius* routinely lick all individuals they come into contact. If the encountered animal is a female in a breeding condition, the male starts courting (Mason & Gutzke 1990). First, male typically performs a stiling posture with all four legs extended and body

elevated above the substrate. The stiling posture is usually accompanied by tail vibrations (LaDage & Ferkin 2006) that are expressed only in the courting context in this species (Brillet 1990). Following, there is chemical examination from distance and closer proximity, the male then starts slowly, cautiously approaching the female and gently bites her flank or tail (described also by Brillet 1990, Brillet 1993). Sometimes the male moves up the female's back (LaDage & Ferkin 2006), grabbing her by the back, neck, or head and simultaneously moves his body parallel to hers (Gutzke & Crews 1988). After these courting phases, the male attempts mounting and if the female allows it (see female mating behavioural pattern), male starts copulating with her (this includes a cloaca contact, intromission, and ejaculation). However, in some cases (see below), the male bites the female in an aggressive context and clearly attacks her.

In our experiments, all males of both species showed interest and almost all of them used a tongue flicking directed to the scent marks on the substrate or directly to various parts of the female body. During mating, the males in our experiments performed longer bites of females in a courting context (mean duration of biting 73.11 ± 9.99 SE) than tail vibrations (mean duration of tail vibration 15.13 ± 1.38 SE); see Supplement 2. We also recorded more attacks on the female during courting of *E. angramainyu* males (in intra- as well as inter- species mating interactions) than *E. macularius* males; see Supplement 2. These male attacks increased the female's refusing behaviour.

We also recorded freezing behaviour that occurs in distinct phases of courting and in a various sequence, probably either reflecting a reaction to the behaviour preceding freezing (e.g. approaching-freezing, vibration-freezing, biting the female in the context of courting-freezing) or as a behaviour preceding the male's decision to behave aggressively towards the female (e.g. freezing-attacking the female). Freezing behaviour in *E. macularius* was also a part of the specific antipredator tactic probably directed to red

sand boa *Eryx johni*, a dangerous sympatric predator with subterranean activity (Landová et al. 2016). Freezing behaviour in leopard gecko thus probably reflects an emotional state in multiple situations, not only during mating.

Female sexual behaviour

From the proximate point of view, female mating behaviour (her receptivity, i.e. willingness to accept a male) relies on her reproductive cycle. Rhen et al. (2000) determined that females were not receptive during early vitellogenesis (20% of receptive females) and were most receptive during late vitellogenesis (80% of receptive females). These stages corresponded with the circulation of progesterone and estradiol plasma levels and with increasing size and visibility of eggs inside the abdominal cavity. Thus, the reproductive status can be determined easily, because follicles and eggs are visible through the abdominal wall (for details see Rhen et. al. 2000). Females are reproductively active since the early to late vitellogenesis for about 15 days. We set our mating experiments to this period. During this period, females also willingly accepting males (Rhen et al. 2000, LaDage and Ferkin 2006). A female ovulates roughly 9 days after reaching the late vitellogenic stage and lays her eggs another 11 days later. After the ovulation, females are not receptive. However, it is not only the female reproductive status influences her decision to accept a male. *E. macularius* females have also ability to store the sperm for a long time (one reproductive season in our stock) and clearly benefit from multiple mating in terms of more viable and hatched eggs (LaDage et al. 2008). Interestingly, there was no difference in the female's willingness to copulate with the previous versus novel mate, however, smaller females, more than the bigger ones, were selective in accepting the familiar and novel partner (LaDage and Ferkin 2007).

If the female reacts positively to the male presence, she usually stays and allows him to bite her tail or neck. Thus, receptive females remain stationary when contacted by

a courting male (Gutzke and Crews 1988). If the female decides to accept courting, she further allows the male to grip her neck and mount on her back. Then she lifts her tail aside allowing the male to place his cloaca to hers and successful copulate. If the female is not receptive or decides to refuse the male, she will terminate the courting by fleeing or biting him.

In our experiments, almost all the females were finally interested in the male courting behaviour (see Supplement 1), however, the relatively short periods when females pay attention to the male (tongue flicking, approaching the male, crossing over the male's body) were interrupted by extended periods of unconcern (outside the mounting and copulation context) with the male presence or by female freezing. If the female refused the male both animals showed apparent unconcern with each other's presence, usually resting or sleeping keeping some distance between them. However, we also recorded females biting the male in an aggressive context. In our experiments, this female behaviour was more frequent in *E. angramainyu* (and also in hybrids) than in *E. macularius* (see Supplement 1).

Behavioural elements/ variables

We recorded 9 elements of male epigamic behaviour. Some elements of sexual behaviour in males like (1) duration of unconcern; (2) tongue flicking (frequency); (3) duration and latency of approaching; and (4) freezing defined the same way as in females (see below). Specifically for males, we recorded elements of male courting and mating behaviour: (5) tail vibration (latency, duration) - the tail is wiggled quickly from side to side, the male is usually in the stilting posture; (6) biting the female in a sexual context (latency, frequency, duration), the male bites the female gently in her tail, back, neck, or the head; (7) attempted copulation (duration), the male is parallel to the female and tries to lift the female's tail by his tail for the free access to copulation; (8) ejaculation

(latency, duration) the animals have a cloaca contact and mating connection, contraction of pelvic muscles is clearly visible and denotes the time of ejaculation; (9) the male attacks the female and bites her in a clearly aggressive context (frequency).

For females, we recorded 8 following elements of behaviour: (1) unconcern (duration), the female has no interest in other individuals, is resting and/or sleeping; (2) tongue flicking (frequency), the tongue samples the substrate-bound, airborne chemicals in the environment as well as semiochemicals from her partner's skin and delivers them to the vomeronasal organ above the roof of the mouth; (3) approaching (latency, duration), the female shows interest in the male, approaches him at least at a distance of 10 cm, observes him and flicks the tongue during this interaction; (4) freezing (duration), the female remains motionless with her ventrum pressed against the floor or stands in a high posture; (5) allowing copulation (latency, duration, presence of behaviour), the female lifts her tail during the male's attempt to copulate; (6) female refusing the male (latency, duration), the female tries to escape from the male, bites him or horizontally waves the tail; (7) the female attacks the male in an aggressive context (presence of behaviour); (8) whether the female allows copulation or not corresponds with the male's copulation success, coded only if successful copulation occurs or not (binary coded data), see Tab. 1.

Parental species/forms and their hybrids and backcrosses

For interspecific hybridization, we recorded sexual behaviour of both parental species *E. macularius* (species: M, cross: MxM) and *E. angramainyu* (species: A, cross: AxA), behaviour during hybridization (female: M, male: A, cross: MxA) and behaviour of hybrids (hybrid: MA, cross: MAXMA). In abbreviation of crosses (e.g. MxA) the first place denotes the female's form (M, *E. macularius* female), followed by the male's form

(A, *E. angramainyu* male). The number of mating interactions per each cross is provided in Supplements 1 and 2.

For hybridization among forms we use: "yellow" form of *E. macularius* (form: M, cross: MxM), "dark" form of *E. macularius* (form D, cross: DxM), their reciprocal hybrids of the first (F1, crosses: MxD or DxM) and second (F2 hybrids, cross: MDxMD) filial generations. Backcrosses of F1 males with "yellow" form females (backcross: MxMD). The reciprocal backcrosses of F1 males with "dark" form females of *E. macularius* (backcross: DxMD) were not carried out because the "dark" form is rare. The above-mentioned generations and/or crossings refer to the embryos and hatchlings, the type of cross denotes the type of mating interaction. In abbreviation of crosses (e.g. MxD), the first place denotes the female's form (M "yellow" form female), the second is the male's form (D "dark" form male). We recorded sexual behaviour in these crosses: MxM, DxM, MxD.

Egg hatching success and juvenile survival in yellow and dark form of E. macularius

During the egg-laying season (since February to September), we controlled the egg-deposition containers three times a week. The eggs were weighted and placed in the temperature controlling incubator in plastic boxes, each containing a single clutch. We set the temperature to $28.5 \pm 0.5^{\circ}\text{C}$, which is an optimal and preferred incubation temperature of *E. macularius* (Bull et al. 1988, Bragg 2000, Landová et al. 2013). For every egg, we took the parents' identity, date of laying and hatching, weight of the egg and hatchling, and the incubation temperature. In order to perform formal tests of hatchability, we used ANOVA for a binomial distribution, in which hatching of the incubated eggs of an individual clutch (number of hatchlings of one clutch and number of non-hatched eggs of the same clutch) was given as a dependent variable with a binomial distribution and the juvenile form as an explanatory variable. Similarly, we measured and

calculated the surviving rate of hatched juveniles up to one year. The hatchlings were weighted and scanned (a ventral and dorsal view of the body) in standardized positions. This procedure was repeated in adulthood at the age of 2–3 years. We assessed egg hatchability and survival of juveniles in parental yellow and dark forms, their reciprocal F1 hybrids, F2 hybrids and backcross with the yellow form.

Methods of statistical testing

First, we employed a Kruskal-Wallis test to check for the effect of cross type (crosses: MxM; AxA; MxA; MAxMA; MxD; DxM) on the original non-transformed variables of particular male and female sexual behaviour. Significant comparisons were further compared by a post hoc Kruskal-Neményi test (Nemenyi, 1963) as implemented in PMCMR package (R-project, R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>). Next, the data were transformed to achieve normality and when needed, they were corrected of unequal duration of mating interaction (successful interactions were shorter). Next, we performed a multivariate canonical discrimination function analysis (DFA) to assess the pattern of differences between intraspecific and interspecific crosses of *E. macularius* and *E. angramainyu* species and their hybrids in the evaluation of all mating behaviours. We performed this analysis separately for each sex. Differences in sexual behaviour between the forms were calculated analogically. To calculate how specific male courting behaviour involves female behaviour, we applied several methods: 1) to quantify the proportion of variation in female behavioural traits which is explained by the male behavioural traits (redundancy) and reveal correlations between the set of female and male behavioural variables, we applied a canonical analysis (CA); 2) to calculate the effectivity of particular male courting behaviour on variables that mostly reflect female receptivity (duration of female allowing copulation), we employed a multiple regression; 3) to calculate which male behaviours could explain successful

copulation with a female (copulation occurs vs no copulation), we applied a GLM model for binary data. We employed statistical GLM models with the logit link function in R-environment (R-project). The list of behavioural traits in each analysis is given in Tab. 1. To compare the incubation success (hatchability) in paternal forms (M, D) and the available categories of hybrids (MD, DM), we adopted a GLM model for the incubation success (hatchability) and survival rate up to one year. An HSD post-hoc test revealed differences among groups. The survival rate of hatched juveniles up to one year was calculated analogically.

Results

Experiment I: Sexual behaviour during interspecific hybridization between *E. macularius* and *E. angramainyu* and sexual behaviour of hybrids vs conspecific mating patterns

Female courting and mating behaviour

First, using a Kruskal-Wallis test, we analysed whether a particular female behaviour differs during conspecific mating (crosses: **AxA**, **MxM**) and hybridization (cross: **MxA**), or during mating of hybrids (cross: **MAxMA**). Differences between particular crosses were revealed by a Kruskal-Neményi post hoc test. Behaviours that reflect the first part of interaction (such as latency of a female approaching a male and tongue flicking during mating) did not differ between crosses. Females of *E. macularius* were interested during a comparable period in both their conspecific males and heterospecific *E. angramainyu* males, but the duration of approaching behaviour was different dependent on the cross (Kruskal-Wallis chi-squared = 38.141, df = 5, p < 0.0001; **MxM** > **MxA** > **AxA**, see Supplement 1). Approaching females of *E. macularius* spent twice as long focusing on males compared to females of

E. angramainyu in intraspecific mating (crosses: **MxM** vs **AxA**; $p < 0.0001$). A similar pattern was found for *E. macularius* females approaching *E. angramainyu* males during interspecific hybridization (crosses: **MxA** vs **AxA**; $p = 0.0251$).

Females' defensive and refusing behaviours like biting the male (Kruskal-Wallis chi-squared = 22.577, $df = 5$, $p = 0.0004$), duration of refusing the male (Kruskal-Wallis chi-squared = 40.425, $df = 5$, $p < 0.0001$), or duration of female freezing (Kruskal-Wallis chi-squared = 60.318, $df = 5$, $p < 0.0001$) were significantly different in some crosses. According to the Kruskal-Neményi post hoc test, the behaviour of females of both studied species differed significantly during intraspecific mating (cross: **MxM** vs **AxA**; refusing the male: $p < 0.0001$; female freezing: $p = 0.0008$). The behaviour of *E. macularius* female during hybridization differ as well (cross: **MxM** vs **MxA**; refusing the male: $p = 0.0001$; female freezing: $p < 0.0001$). Differences in behaviour were even found in hybrids (cross: **MAxMA**; refusing the male: $p = 0.0009$; female freezing: $p = 0.005$). Half of the *E. angramainyu* females (cross: **AxA**) and hybrid females (cross: **MAxMA**) attacked and bit the male during refusing, while only a small proportion (16%) of *E. macularius* females bit the male during intraspecific mating or hybridization (18%; **MxA**), see Supplement 1.

Crosses also differ in their behaviour reflecting female receptivity (latency to allow copulation: Kruskal-Wallis chi-squared = 71.058, $df = 5$, $p < 0.0001$; duration of copulation allowed by female: Kruskal-Wallis chi-squared = 29.014, $df = 5$, $p < 0.0001$) or indifference (duration of unconcerned behaviour: Kruskal-Wallis chi-squared = 59.25, $df = 5$, $p < 0.0001$). Kruskal-Neményi post-hoc test revealed differences in behaviour of *E. macularius* (cross: **MxM**) and *E. angramainyu* females during conspecific mating (cross **AxA**; latency to allow copulation: $p < 0.0001$; duration of allowed copulation: $p = 0.0092$; duration of unconcerned behaviour: $p = 0.0235$). Behaviour of *E. macularius*

females during conspecific mating and during hybridization differs as well (crosses: **MxM** vs **MxA**; latency to allow copulation: $p < 0.0001$; duration of allowed copulation: $p = 0.04$, duration of unconcerned behaviour: $p = 0.0003$), and there are differences in behaviour of hybrid females (crosses: **MxM** vs **MAxMA**; latency to allow copulation: $p = 0.0002$; duration of unconcerned behaviour: $p = 0.0003$). More precisely, half of the females of *E. macularius* allowed copulation, but only 15 % of *E. angramainyu* females accepted the male of their own species. Similar proportion of hybrid females (cross: **MAxMA**; 18%;) allowed copulation. The number of females that allowed copulation during hybridization was even lower, only 13% of *E. macularius* females accepted a male of the other species (cross: **MxA**). During conspecific mating (**MxM** and **AxA**) females of both species allowed copulation earlier than did females of *E. macularius* during hybridization (**MxA**). However, there are interspecific differences in duration allowing copulation, it is longer in *E. angramainyu* and hybrid females (cross: **MAxMA**) than in *E. macularius* females (see Supplement 1).

Next, we performed a multivariate canonical discrimination function analysis (DFA) to assess the pattern of differences between intraspecific and interspecific crosses of *E. macularius* and *E. angramainyu* species and their hybrids in the evaluation of all mating behaviours of females ($N = 135$ interactions, Wilks' lambda = 0.1666, $F_{(30,358)} = 10.07$, $p < 0.0001$). The forward stepwise procedure selected 10 of 12 behavioural traits (latency of tongue flicking and duration of approaching to the male were not included in the model). The variables corresponding the best with the discriminatory criteria (i.e. the largest Wilks' lambda) were latency to allow copulation (Wilks' lambda = 0.2203, $p < 0.0001$), duration of female unconcern (Wilks' lambda = 0.2027, $p < 0.0001$), and frequency of female tongue flicking (Wilks' lambda = 0.2019, $p < 0.0001$). The total classification success was 74.81%. For

detailed classification success of female mating behaviour in particular crosses see Tab. 2.

The pattern of mating behaviour of *E. macularius* females was different from that expressed by *E. angramainyu* females ($F_{(10,122)} = 21.97$, $p < 0.0001$). During hybridization, when *E. macularius* females were crossed with heterospecific *E. angramainyu* male (cross: **MxA**), female mating behaviour was different from all the other crosses: conspecific mating in *E. macularius* (cross: **MxM**; $F_{(10,122)} = 21.69$, $p < 0.0001$), in *E. angramainyu* (cross: **AxA**; $F_{(10,122)} = 7.13$, $p < 0.0001$), and mating of the hybrids (cross: **MAxMA**; $F_{(10,122)} = 3.34$, $p = 0.0007$, see Fig. 1). Next, we performed a canonical variance analysis (CVA) to detect the most discriminating variables. First axis discriminates mostly according to female's willingness to copulate (duration as well as latency to allow copulation) and female unconcern with the male. The second axis discriminates according to female freezing behaviour or active refusal of the male and again according to female allowance of copulation (loadings are provided in Tab. 3).

Male courting and mating behaviour

In males, we first analysed by a Kruskal-Wallis test whether a particular male behaviour differs interspecifically (crosses: **AxA** and **MxM**), during hybridization (cross: **MxA**), or during hybrids mating (cross: **MAxMA**). Differences between particular crosses were then revealed by a Kruskal-Neményi post-hock test. The males differed in copulation success (Kruskal-Wallis chi-squared = 26.202, $df = 5$, $p < 0.0001$). *E. macularius* males were more successful (50%) than *E. angramainyu* males (6%; $p = 0.022$). The male copulation success in other crosses was lower, though insignificantly: in hybridization experiments, only 13% of *E. angramainyu* males and 18% of hybrid males copulated successfully with hybrid females (see Supplement 2). However, all the males were interested in the females during the experiments. Moreover,

no differences were found between crosses either in latency to approach the female or duration of approaching. The males explored the female's presence by vomero-olfaction using tongue flicking differently in each cross (Kruskal-Wallis chi-squared = 20.281, $df = 5$, $p = 0.0011$) and the number of tongue flicks was higher during intraspecific mating of *E. macularis* (cross: MxM) compared to *E. angramainyu* (cross: AxA; $p = 0.0108$) and during hybridization (cross: MxA; $p = 0.0022$).

In all the experiments, males showed a similar duration of unconcerned behaviour and devoted a similar proportion of time to courtship. The duration of tail vibration and biting the female in a sexual context did not differ between crosses. However, latency to the first vibration (Kruskal-Wallis chi-squared = 14.863, $df = 5$, $p = 0.0109$) and ejaculation (Kruskal-Wallis chi-squared = 28.872, $df = 5$, $p < 0.0001$) were different. Males of *E. macularius* vibrated earlier than *E. angramainyu* in hybridization experiments (crosses: MxM vs. MxA; $p = 0.025$). The males of *E. angramainyu* ejaculated earlier than *E. macularius* in conspecific mating experiments (crosses: AxA vs. MxM; $p = 0.0097$), but not during hybridization when males of the former species *E. angramainyu* took longer to ejaculate when mating with *E. macularius* female (crosses: MxA vs. MxM; $p = 0.0466$), see Supplement 2. The duration of ejaculation differed between forms (Kruskal-Wallis chi-squared = 29.771, $df = 5$, $p < 0.0001$) in a similar manner (crosses: MxM vs. AxA; $p = 0.01$; crosses: MxA vs. MxM; $p = 0.0430$). The frequency of male attacks on females was very rare in *E. macularius* males (6% of males) compared with *E. angramainyu* males (cross: AxA) or male hybrids (crosses: MAxMA), where 23 or 29 % of males attacked the female, respectively. Only 13% of *E. angramainyu* males attacked *E. macularius* females during hybridization (cross: MxA), see Supplement 2.

Next, the analogical canonical DFA of all the male mating behaviours (Wilks' lambda = 0.61; $F_{(12,338)} = 5.78$; $p < 0.0001$) revealed that the overall reclassification success was very low (45.19%, for details see Tab. 4). In the analysis, the number of included behavioural traits was reduced by a forward stepwise procedure to just 4 out of a total of 12 traits (latency of approaching the female, tongue flicking, tail vibration, and biting the female in a sexual context; duration of approaching the female, freezing, tail vibration, and ejaculation were not included in the model). The variables corresponding the best to the discriminatory criteria were the latency of ejaculation (Wilks' lambda = 0.7270, $p < 0.0001$), frequency of male tongue flicking (Wilks' Lambda = 0.6804, $p = 0.0031$), duration of male unconcern with the female (Wilks' Lambda = 0.6830, $p = 0.0025$), and duration of biting the female in a sexual context (Wilks' lambda = 0.6686, $p = 0.0090$).

The males of both species significantly differed in their pattern of sexual behaviour ($F_{(4,128)} = 8.65$, $p < 0.0001$), similarly to that of females. Mating behaviour of *E. angramainyu* males during hybridization with a female of *E. macularius* differed the most from that of *E. macularius* males during mating with a conspecific female (crosses: MxA vs. MxM; $F_{(4,128)} = 10.65$, $p < 0.0001$). A smaller difference in behaviour between males of the *E. angramainyu* species was found when *E. angramainyu* was mating with a conspecific female and when was mating with heterospecific females (crosses: AxA vs. MxA; $F_{(4,128)} = 4.54$, $p = 0.0018$). However, when we compared *E. angramainyu* males during hybridization with the hybrid males (MA) mating with the hybrid females, there was no significant difference (crosses: MxA vs. MAxMA; $F_{(4,128)} = 0.94$, $p = 0.4443$). A plot of the first two canonical factors showed a considerable overlap of the groups (see Fig. 2, loadings are provided in Tab. 5).

Experiment II. Sexual behaviour during hybridization between “yellow” and “dark” form

Female courting and mating behaviour

In the second experiment, we observed mating behaviour of two forms; the “yellow” *E. macularius* (M) and “dark” (D) form. To test differences between forms in particular female behaviour we compared: behaviour of “yellow” form during conspecific mating with males of the same form (cross: **MxM**) or during hybridization with males of the “dark” form (cross: **MxD**). Furthermore, we also observed behaviour of the “dark” form females during hybridization with a “yellow” form male (cross: **DxM**). We used a Kruskal-Wallis test to reveal differences in each behaviour and a Kruskal-Neményi post-hoc test to quantify differences between crosses.

There was a big difference in duration of female refusing behaviour between crosses (Kruskal-Wallis chi-squared = 41.441, df = 5, $p < 0.0001$), which might be explained by the fact that the “yellow” females frequently accepted males of both the same (cross **MxM**; 50%) and different form (cross: **MxD**; 40%), however, the “dark” form females accepted the “yellow” form males only rarely (cross: **DxM**, 13%), see Supplement 1. The “dark” form females refused males during hybridization longer than the “yellow” form females mating with a male of the same form (crosses: **DxM** vs. **MxM**; $p < 0.0001$) or during hybridization with a “dark” form male (crosses: **DxM** vs. **MxD**; $p = 0.0225$). There were also significant differences in frequency of female biting the male during refusing (Kruskal-Wallis chi-squared = 22.577, df = 5, $p = 0.0004$), but the crosses did not significantly differ one from another according to Kruskal-Neményi post hoc test. Similarly, duration of unconcern with the male in the “yellow” form females differed the same way as female refusing behaviour (Kruskal-Wallis chi-squared = 59.25, df = 5, $p < 0.0001$). In the “yellow” form females the durations of unconcern

with a “dark” form male during hybridization were longer than in trials with a male of the same form (crosses: **MxD** vs. **MxM**; $p = 0.0001$, which was also confirmed in the “dark” females (crosses: **DxM** vs. **MxM**; $p = 0.00014$). The latency with which the “yellow” females allowed copulation with their own males differed compared to mating with the “dark” form males (Kruskal-Wallis chi-squared = 71.058, $df = 5$, $p < 0.0001$). When a “yellow” female hybridized with a “dark” male, she allowed copulation later (crosses: **MxD** vs. **MxM**; $p < 0.0001$); the similar pattern was found for the “dark” form females (crosses: **DxM** vs. **MxM**; $p = 0.0227$). Both the “yellow” and “dark” females froze for a different proportion of time depending on the crosses (Kruskal-Wallis chi-squared = 60.318, $df = 5$, $p < 0.0001$). The “yellow” females froze longer when mating with the males of same form than when hybridizing with the “dark” form males (crosses **MxM** vs. **MxD**; $p < 0.0001$). The “dark” females froze shorter during hybridization with a “yellow” male than “yellow” female during mating a same form male (crosses: **DxM** vs. **MxM**; $p = 0.0002$).

Analogically to the first interspecific hybridization, we performed a canonical discrimination function analysis (DFA) to assess intraspecific differences between forms ($N = 74$ interactions, Wilks’ lambda = 0.1729, $F_{(12,132)} = 15.45$, $p < 0.0001$). The forward stepwise procedure selected 6 out of 12 behavioural traits. The variables corresponding the best to the discriminatory criteria (i.e. the largest Wilks’ lambda) were the duration of freezing (Wilks’ lambda = 0.3618, $p < 0.0001$), latency to allow copulation (Wilks’ lambda = 0.2904, $p < 0.0001$), duration of allowed copulation (Wilks’ lambda = 0.2892, $p < 0.0001$), and the duration of active refusing of the male (Wilks’ lambda = 0.2069, $p = 0.0027$). The total classification success was 85.14%. For a detailed classification success of female mating behaviour in individual crosses see Tab. 6.

During hybridization, when the “yellow” form females of *E. macularius* were crossed with a “dark” form male of the same species (crosses: **MxD** vs. **MxM**; $F_{(9,66)} = 27.00$, $p < 0.0001$), or when the “dark” form females were crossed with a “yellow” form male (crosses: **DxM** vs. **MxM**; $F_{(9,66)} = 25.68$, $p < 0.0001$), female mating behaviour differed from control mating of “yellow” forms of *E. macularius* (see Fig. 3). In the canonical variance analysis (CVA), the first axis discriminates mostly according to the female’s willingness to copulate (duration as well as latency to allow copulation) or according to the behaviour reflecting fear (female freezing behaviour). The second axis discriminates according to the active refusing of the male and again according to the female’s allowance of copulation (loadings are provided by Tab. 7).

Male courting and mating behaviour

To test intraspecific differences in particular male courting and mating behaviours, we compared: behaviour of a male from the “yellow” population of *E. macularius* during intraspecific mating with a female of the same form (cross: **MxM**) or during hybridization with a female of the “dark” form (cross: **DxM**) with behaviour of a “dark” form male mating with a “yellow” female (cross: **MxD**). We used a Kruskal-Wallis test to reveal differences in each behaviour and a Tukey’s post-hock test to quantify differences between crosses (**MxM** vs. **DxM** vs **MxD**).

The males were interested in their own females as well as in the “dark” form females. There were no differences between crosses in the tongue flicking (latency, frequency), approaching the female (latency, duration), tail vibration (latency, duration), biting the female in a sexual context (frequency), biting the female in an aggressive context (binary coded), ejaculation (latency, duration), and copulation success (binary coded). Only the behaviours possibly reflecting the male emotional state (duration of freezing) or motivation to mate (duration of unconcern) differed between forms. Duration

of male freezing differed between intraspecific crossing of “yellow” (cross: MxM) and “dark” (crosses: MxD and DxM) forms (Kruskal-Wallis chi-squared = 25.079, df = 5, $p = 0.0001$). The “yellow” males froze longer when mating with their own female than during hybridization with a dark female (crosses: MxM vs. DxM; $p = 0.0005$). In the contrary, the “dark” form males froze for a shorter period when mating a “yellow” female than did “yellow” males during hybridization with “dark” female (crosses: DxM vs. MxD; $p = 0.0341$). The duration of unconcerned behaviour is the second parameter of male behaviour that differed between crosses during intraspecific mating (Kruskal-Wallis chi-squared = 18.068, df = 5, $p = 0.0029$). Duration of unconcern was longer when “yellow” males mated the other form female during hybridization than when mated their own females (crosses: DxM vs. MxM; $p = 0.0492$). Males of the “dark” form expressed different level of unconcern during hybridization as well (crosses: MxD vs. MxM; $p = 0.0140$).

In the DFA of all the male mating behaviours (Wilks’ lambda = 0.32; $F_{(18,126)} = 5.32$; $p < 0.0001$), the forward stepwise procedure selected 9 of 12 behavioural traits (the latency and duration of approaching the female and the latency of biting her were not included in the model). The variables corresponding significantly with the discriminatory criteria were the duration of unconcern with the female (Wilks’ lambda = 0.3855, $p = 0.0037$), latency of male tail vibration (Wilks’ Lambda = 0.3750, $p = 0.0089$), duration of ejaculation (Wilks’ lambda = 0.3714, $p = 0.0122$), and duration of male freezing behaviour (Wilks’ lambda = 0.3675, $p = 0.0169$). The reclassification success was 75.68% (for details see Tab. 8).

As with the females of *E. macularius*, mating behaviour of the “yellow” form males during pairing with conspecific females differed from those crossed with the “dark” form of *E. macularius* females (crosses: MxM vs. DxM; $F_{(9,63)} = 7.59$,

$p < 0.0001$) and from the “dark” form males being crossed with the “yellow” form females (crosses: MxD vs. MxM; $F_{(9,63)} = 3.72$, $p = 0.0009$, see Fig. 4).

In the canonical variance analysis (CVA), the first axis discriminated mostly according to the male’s unconcern with the female (duration of unconcern) or the duration of ejaculation and male freezing behaviour. The second axis discriminated according to the latency of tail vibration, males’ unconcern about the female, and the duration of ejaculation (loadings are provided by Tab. 9).

Reproduction success of the “yellow” and “dark” form and their hybrids

To compare the incubation success (hatchability) in paternal forms (M, D) and the available categories of hybrids (MD, DM), we adopted a GLM model for the incubation success (hatchability) and survival rate up to one year. An HSD post-hoc test revealed differences among groups. The model revealed a significant variation in incubation success among the examined groups (species and categories of hybrids; $df = 4$, $F = 8.21$, $P < 0.0001$, see Tab. 10). The incubation success of *E. macularius* was significantly higher than that found in every other examined group (forms: M vs. D, $p = 0.0003$; M vs. MD, $p = 0.0011$; M vs. DM $p < 0.0001$). However, the incubation success of the dark form was not different from hybrids of a yellow form female and a dark form male (forms: D vs. MD, NS).

Most of the hatchlings successfully survived up to the age of one year; there was not a significant effect of species/hybrid category at p -level < 0.05 (NS, $F = 2.28$, $p = 0.0807$). Only survival of the dark form juveniles was lower compared to the yellow form at $p < 0.1$ ($p = 0.0941$).

Experiment III: Relationship between male and female sexual behaviour

Total correlation of male and female behavioural pattern

The canonical analysis revealed a significant multivariate correlation between female (9 traits) and male (11 traits) behavioural patterns (canonical $R = 0.911$, $N = 177$ pairs, Chi-Square = 633.20, $df = 99$, $p < 0.0001$, Fig. 5). Redundancy given by the set of male behavioural traits is 37.8%, it quantifies the proportion of variation in female behavioural traits which is explained by the male ones. Correlations of behaviours are given in Tab. 11. Behaviour reflecting female receptivity (female unconcern) correlates highly with the male unconcern with mating (0.71). Male behaviours preceding a successful copulation highly correlate with the female decision to allow copulation (biting the female in a sexual context 0.77, mating attempt 0.75).

Male behaviours involving the female decision to accept the male

We can also determine which male behaviours affect female behaviours preceding successful copulation. If the female is receptive, she lifts her tail and allows the male to join and copulate. A multiple regression revealed that the female's decision to allow copulation expressed as time devoted to this behaviour is explained by the model ($R^2 = 0.65$, $F_{(6,171)} = 52.07$, $p < 0.0001$) and is negatively correlated with the time of male approaching the female ($t = - 7.82$, $p < 0.0001$), male freezing ($t = - 5.87$, $p < 0.0001$), and male unconcerned behaviour ($t = - 5.33$, $p < 0.0001$). When males spent more time by biting the female ($t = 5.29$, $p < 0.0001$) or did more tongue flicking ($t = 3.21$, $p < 0.0016$), then the female's decision to allow copulation was positively correlated and the males had better chance to mate (Fig. 6).

To calculate which male behaviours could explain successful copulation with the female (copulation occurs vs no copulation), we applied a GLM model for binary data. We employed statistical GLM models with the logit link function in R-environment

(R-project) with the type of crossing and male behaviours (tongue flicking, unconcern, approaching, freezing, vibration, biting of females) as a fixed effect. The best fitting model according to the AIC criteria includes all the factors excluding tongue flicking. The type of cross (MxM, AxA, MxA, MAxMA, DxM, MxD, $p = 0.0002$), male unconcern ($p < 0.0001$), approaching ($p < 0.0001$), freezing ($p < 0.0001$), and biting the female ($p = 0.0067$) had all a significant effect, only vibration had not ($p = 0.417$). Conspecific mating behaviour within *E. macularius* (cross MxM) was different from the other crosses on the level of significance $p < 0.1$ (z value = 1.77, $p = 0.0762$, see Tab. 12), i.e. marginally significant.

Discussion

The effect of allopatric isolation on distant interspecific hybridization

Allopatric reproductive isolation is not directly favoured by selection, but is a secondary consequence of genetic differentiation driven by selection on other traits. Allopatric species face ecological and ethological (competition, predation) conditions in nature consequently leading to body size and behavioural differences (e.g., in stickleback, McKinnon et al. 2004, Rundle et al. 2013, for review, see Funk et al. 2006). These differences, mainly in sexual behaviour, may serve as precopulation reproduction isolation mechanisms in the case of a future secondary contact of the species (Schluter 2001) or under laboratory conditions (McKinnon et al. 2004, Vines and Schluter 2006). Females, rarely also males, chose subtle behavioural traits of conspecifics of the other sex, which can automatically result in discrimination against heterospecific mates (Writz 1999). Discrimination against heterospecific mates is further associated with specific fitness cost (Nagel and Schluter 1998, Rundle and Schluter 1998).

Despite more than 12-15 million years of isolation between *E. macularius* and *E. angramainyu* and the fitness cost of hybridization in forming second-generation hybrids (Jančúchová-Lásková 2005b), the differences in sexual behaviour do not lead to a complete isolation between our studied species as hybridization may frequently occur. Sexual behaviour of mates during hybridization has an intermediate character. There are some quantitative differences during interspecific hybridization: the vomeroolfaction through tongue flicking is higher, especially in females and males ejaculate later when mating with heterospecific females, because females allow copulation later. However, heterospecific *E. macularius* females devote shorter time to allow copulation (duration) with a male of *E. angramainyu* compared to bigger *E. angramainyu* females during intraspecific mating. Moreover, females of the two species show a different level of motivation to mate. Interspecific differences in the level of female aggressive behaviour when refusing the male, result in asymmetry in female willingness to mate during hybridization and cause an asymmetric gain per each sex. Females of the bigger species, *E. angramainyu*, actively refuse courting males, frequently bite them and show high fear reflecting behaviour (freezing) during intraspecific mating. On the other hand, smaller *E. macularius* females during hybridization show lower frequency of freezing than during intraspecific mating and bite the conspecific as well as heterospecific male only rarely during refusal. A male of the bigger species devotes similar energy to courting with a female of own species as well as with a heterospecific female, however, mating with a smaller female during hybridization is less risky for him if the female is not receptive. We did not perform mating between a female of *E. angramainyu* (the rare species) and a *E. macularius* male, so we can only speculate that mating with females showing high level of aggression during intraspecific mating would be difficult for them.

Asymmetric hybridization, when females of one species are more likely to mate with males of the other species than vice versa is quite common in nature (Writz 1999, Rosenthal 2013). Usually those asymmetries can arise from the situation when individuals of one species are generally more attractive to those chose (Stein and Uy 2006, Dame and Petren 2006). This is not exactly the situation in our geckos; the pattern of sexual behaviour during hybridization may contribute to the preference mainly in males that may choose less aggressive partner for mating. Similar situation was found in the stickleback, where in hybrid conditions males preferred for mating smaller females that were not aggressive and did not eat eggs in male's nest (Nagel and Schluter 1998). Sometimes, ecological conditions affect mating decisions in favour of hybridization in one species. In our studied model, the interspecific hybrids are bigger than *E. macularius* species, but smaller than *E. angramainyu* (Jančúchová-Lásková 2015b), and consequently, the growth of hybrids is finished earlier than in *E. angramainyu* (Lásková 2008 master thesis, unpublished data). The bigger body size in eublepharid geckos brings a direct advantage in male-male aggressive interactions (Kratochvíl and Frynta 2002). There may also be an advantage in foraging behaviour; a similar effect of widening the foraging niche in hybrids was demonstrated in spadefoot toad hybrids (Pfenning et al. 2007). In our laboratory conditions, the bigger species and hybrids can include small vertebrates into the diet and get more energy and various nutrients for growth. However, an increase in body size in hybrid offspring is advantageous only for *E. macularius*. The second parental species, *E. angramainyu* may theoretically benefit from the fact that hybrids reach the adult body size earlier (Frynta et al. in prep.). This may be advantageous under a strong predation pressure (Pfenning 2007) as the tactic to avoid predation differs between the young and adult stage in eublepharids (Landová et al. 2013). The situation when a faster growth rate of hybrids outweighs the disadvantage of

lower average fitness possessed by hybrids was described in spadefoot toads. *Spea bombifrons* females in shallow rapidly drying ponds benefit from hybridization with *S. multiplicata*, because the hybrids develop more rapidly than *S. bombifrons* and can escape from drying ponds and therefore survive. In this set of conditions, females of spadefoot toads mate preferentially with heterospecifics and this advantage of hybridization is also asymmetric (Pfennig 2007).

Behaviour of interspecific hybrids and forming backcrosses

For hybrids, future reproduction backcrossing with one of the parental species is one of the options, whenever the species has to face the problem with reproduction in latter generations (e.g., in *Drosophila*: Noor et al. 2001, copepods: Elison and Barton 2008), especially in lizards (Jančúchová-Lásková 2015a). In our experimental system of distant hybridization, when the hybrids can reproduce mainly via backcrossing with one of the parental species (0% reproduction success with *E. angramainyu*, but 40 to 75% egg hatchability in reciprocal mating with *E. macularius*), the pattern of hybrid sexual behaviour plays a key role in reproductive success. For the hybrids, choosing *E. macularius* as a mating partner will maximize reproductive success, while choosing the other species reduces it substantially. Preference for a hybrid partner will lead to a pure fitness loss for hybrids, because hatchability of hybrid eggs is low (6 %) and F2 hybrids have morphological malformations and low viability (see Jančúchová-Lásková 2015a). If we suppose that similarity in sexual behaviour pattern also leads to successful mating and preference for the most similar partner, then sexual behaviour of hybrids is consistent with a potential reproductive isolation of our examined species. Unfortunately, the hybrid females show most similarities in qualitative and quantitative behavioural traits to that of *E. angramainyu*. The hybrid females devoted a high proportion of time to active refusal and more than half of them bit the male in an aggressive context such as

E. angramainyu females. Moreover, the hybrid females also allowed the copulation later compared to females of both parental species and spent more time allowing copulation than *E. macularius* females. However, both sexes expressed less freezing, reflecting a low level of fear during mating. In the system where females have a predisposition gain by a similarity in sexual behaviour to mate with *E. angramainyu* as successfully as with their own hybrid males, the potential for a female mating with a wrong partner is high. The maladaptive preference of hybrid females for their own sterile hybrid males over pure parental species (backcrosses are possible) was reported in hybrids of spadefoot toads *Spea multiplicata* and *S. bombifrons*. However, this preference was also influenced by ecological factors and population characteristics in natural habitats (Schmidt and Pfennig 2016).

The hybrid males have an intermediate character of sexual behaviour pattern with a higher variability in sexual behaviour that allows them to mate successfully with *E. macularius* females. In our previous experiment, backcrosses with a hybrid male and *E. macularius* females had higher hatchability (cross MxMA: 75%) than reciprocal backcrosses (cross MAxM: 40%) possessing additional advantage. However, when unidirectional backcrosses are formed, they may reproduce with both parental species again (Jančúchová-Lásková 2015b). As has been already shown in eublepharids, both sexes of hybrids can benefit from multiple mating (La Dage 2008, our unpublished data) and also from the bigger body size than one of the parental species. It gives the males the advantage in male-male competitions and reduced freezing behaviour of hybrids as well as smaller *E. macularius* females during hybridization may favour this combination of partners. Similarly, in the swordtail fish, females that prefer large body size are less likely to discriminate against heterospecific males (Rosental and Ryan 2011)

The effect of small genetic divergence on differences in sexual behaviour between forms

Our “yellow” and “dark” forms are morphologically and genetically distinct, but the divergence is smaller compared to the divergence between *E. macularius* and *E. angramainyu* (Starostová et al.2005, Jančúchová-Lásková 2015b). We do not know if these populations live in a sympatric or parapatric situation in Pakistan (more probable, see Seuffer 2005) or if they are allopatric for some period of time. For the sympatric or parapatric situation the reinforcement of precopulation RIM was proposed (Dobzhansky1940, Blair 1955 for review see Marshall 2002, Servedio 2004, Svensson et al.2007, Nossil et al. 2006). In this case the premating isolation mechanisms as well as differences in reproduction behaviour should be relatively high because there is relatively high cost of hybridization. In the allopatric situation however, isolation should cause only subtle differences in sexual behaviour and low cost for producing hybrids and hybrid future reproduction as was revealed in current experiments. In this theoretical case, we can suppose that adaptation of the “yellow” of *E. macularius* and “dark” form of *E. cf. fuscus* to slightly different ecological conditions might have caused that the parental species are not genetically and behaviourally divergent enough and premating reproduction isolation has not yet been formed.

We are not able to demonstrate the situation in the field, but we measured the intrinsic cost via measuring egg survival for pure species and reciprocal hybrids as well as differences in survival up to one year. We also precisely measured and quantified the magnitude of differences in sexual behaviour. During close hybridization between the yellow and dark form, we found asymmetry in the fitness cost during hybridization. The yellow form has a lower incubation success when hybridizing with the dark form. However, the dark form hatchability was the same whether the mate was of the same

form or during hybridization between a yellow form female and a dark form male. However, a reciprocal cross (a dark female with a yellow male) was not successful at all. We incubated 51 eggs, but only three juveniles hatched and two survived up to one year. This means that the fitness gain of this hybridization cross is close to zero and thus, becoming a loss for both parental forms. Nevertheless, constraints in hybrids between forms on future reproduction are weaker than constraints in interspecific hybrids. The F1 hybrids can freely reproduce forming F2 hybrids or backcrosses with the “yellow” form and their reproduction success is similar to that of the pure “dark” form. The overall pattern of female sexual behaviour shows not only differences between the pure yellow form and hybridization, but also clear differences between sexual behaviour of the yellow and dark female during hybridization. Similar, but weaker differences were also observed in male sexual behaviour.

The cost of hybridization between distant and close hybridization is higher for the former, however the loss of fitness and possible gains per species are clearly asymmetric. The yellow form of *E. macularius* is more permissive for distant as well as close hybridization between forms. This permissiveness is through the ability of the yellow females to produce F1 hybrids and successfully backcross with these hybrids. As distant interspecific hybridizations may bring not only costs, but theoretically some fitness advantages via bigger body size of the hybrids as well, the close hybridization have only negative effects on eggs hatchability, especially in one direction in our model system. These potential hybrid advantages should be further tested via assessing parameters of growth or by measuring hybrid performances in laboratory or by measuring survival rate in natural conditions. However, in both cases of hybridization there are also patterns of differences in sexual behaviour especially in females that have a potential as a base for

future discrimination among conspecific and heterospecific males and even reciprocal hybrids in some cases.

What male behaviours influence mating success?

The behaviour of males and females is inevitably correlated; however, behaviours that correlate the most are negatively associated with the copulation success and reflect low female receptivity and motivation to mate. However, if the male engages in chemical examination of the female and subsequently intensifies courting (biting female during courting, tail vibration) she will more probably allow copulation. Still, *E. macularius* males have the highest frequency of tongue flicking during conspecific mating and their copulation success was also the best when mating with conspecific females. Tongue-flicking activity is generally considered to reflect a sexual interest and has been used in several previous studies of mate preferences and sexual isolation in reptiles (Shine et al., 2002; Barbosa et al., 2006; Martin and Lopez, 2006). Male leopard geckos use semiochemicals from the skin as sex pheromones and increase the frequency of tongue flicking which is mainly related to vomeroolfaction when discriminating sex (Brillet 1990, Mason and Gutzke 1990) or familiarity of the female (Steele and Cooper 1997, LaDage and Ferkin 2006). Similar chemical communication is frequently used for male perception of female reproductive status and her attractiveness in snakes (reviewed in Martin and Lopez 2011) and may be similarly used by males in leopard geckos.

The hypothesis that chemical stimulus can be the basis of interspecific recognition that may reduce the frequency of hybridization was tested several times in lizards and snakes. The Columbretes Islands wall lizard *Podarcis atrata* and Iberian wall lizard *P. hispanica* chemically discriminate their own and heterospecific individuals and increase tongue flicking in response to own species (Gabriot et al.2009). Chemical species recognition was also shown in other *Podarcis* species (Barbosa et. al. 2006,

Martin and Lopez 2006, Runemark et al.2009). Comparable results were obtained by Labra (2011) for three species of lizards of the genus *Liolemus* living in sympatric or allopatric situation. All species respond more to chemical signals of their own species than those of congeners, which has been also shown in sympatric sea snakes species by Shine et. al (2002). However, in our case the differences in tongue flicking was between species (*E. macularius* males had higher tongue flicking frequency than *E. angramainyu*), but not between the genetically and morphologically distant forms *E. macularius* and *E. cf. fuscus*. More importantly, in both cases hybridization occurs. There are two possible explanations, either the chemicals are too similar in our species to help recognition or chemical signalling is one of many communication ways involved during interspecific mating. The ability to discriminate own species chemically may behaviourally influence only one sex. Male lizards of *Psammodoromus algirus* from distant lineages responded more aggressively toward scent of males of the opposite lineage, but females did not recognize these differences by chemosensory cues and did not prefer males of their own lineage. Thus, these lineages are probably reproductively isolated only partially (Martin et al. 2016). Shine (2004) also found the ability of chemical species recognition in two species of *Thamnophis* snake (*T. sirtalis* and *T. radix*), but at the same time one of the species still freely hybridizes with the other and premating isolation in these two species should be strengthened by different timing of reproduction.

Conclusion

In conclusion, quantitative differences in sexual behaviour are not sufficient to prevent distant or close hybridization of *E. macularius* and its two congeners. In our experimental model of distant and close hybridization, we found asymmetric fitness costs/advantages of hybrids, for each parental species and sex. In distant hybridization, smaller females of *E. macularius* and their offspring may benefit from the bigger body size of interspecific hybrids. On the other hand, males of *E. angramainyu* and hybrid males may benefit from a low risk of female aggression during mating with nonreceptive females and from previous growth deceleration of hybrid offspring. Moreover, sexual behaviour of hybrid females is more like that of, the parental species, *E. angramainyu*, where backcrossing is not possible, which causes them further fitness loss. In the model of close hybridization, males of *E. macularius* “yellow” form have greater fitness loss when mating with the “dark” form of *E. cf. fuscus* females. Furthermore, they did not utilize differences in sexual behaviour of the “dark” and “yellow” form females to avoid heterospecific mating. The differences between species, hybridization events, and hybrids, were more apparent in female sexual behaviour at both levels of hybridization. However, we found more differences in sexual behaviour in distant hybridization, when *E. angramainyu* and hybrids females were clearly more aggressive and less receptive.

Overall, the differences in sexual behaviour are more apparent in the case of distant hybridization compared to the close one, however, though not divergent enough to prevent experimental hybridization. The level of isolation depends not only on genetic, morphological and behavioural differences, but also on the ecological conditions selecting for future differentiation of reproductive isolation between species/lineage in nature. The question how the experimentally revealed differences in sexual behaviour are

linked with advantages and disadvantages in fitness at two levels of hybridization needs further examination under natural conditions.

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Supplements

Supplement 1. The frequencies, latencies and duration time of the female behavioural traits (mean \pm SE are given). The conspecific matings of the *E. angramainyu* (AxA) and the *E. macularius* (MxM), the hybridization between the *E. macularius* female and the *E. angramainyu* male (MxA), the mating between their hybrids (MAxMA), the hybridization between the *E. macularius* female and the “dark” form of the *E. cf fuscus* male (MxD) and the hybridization between the “dark” form female and the “yellow” form male (DxM). n = the observed number of pairing belonging to each category. The means are calculated only where the behaviour present.

Supplement 2. The frequencies, latencies and duration time of the male behavioural traits (mean \pm SE are given). The conspecific mating of the *E. angramainyu* (AxA) and the *E. macularius* (MxM), the hybridization between the *E. macularius* female and the *E. angramainyu* male (MxA), the mating between their hybrids (MAxMA), the hybridization between the *E. macularius* female and the “dark” form of *E. cf fuscus* male (MxD) and the hybridization between the “dark” form female and the “yellow” form male (DxM). n = the observed number of pairing belonging to each category. The means are calculated only where the behaviour present.

Fig. 1.

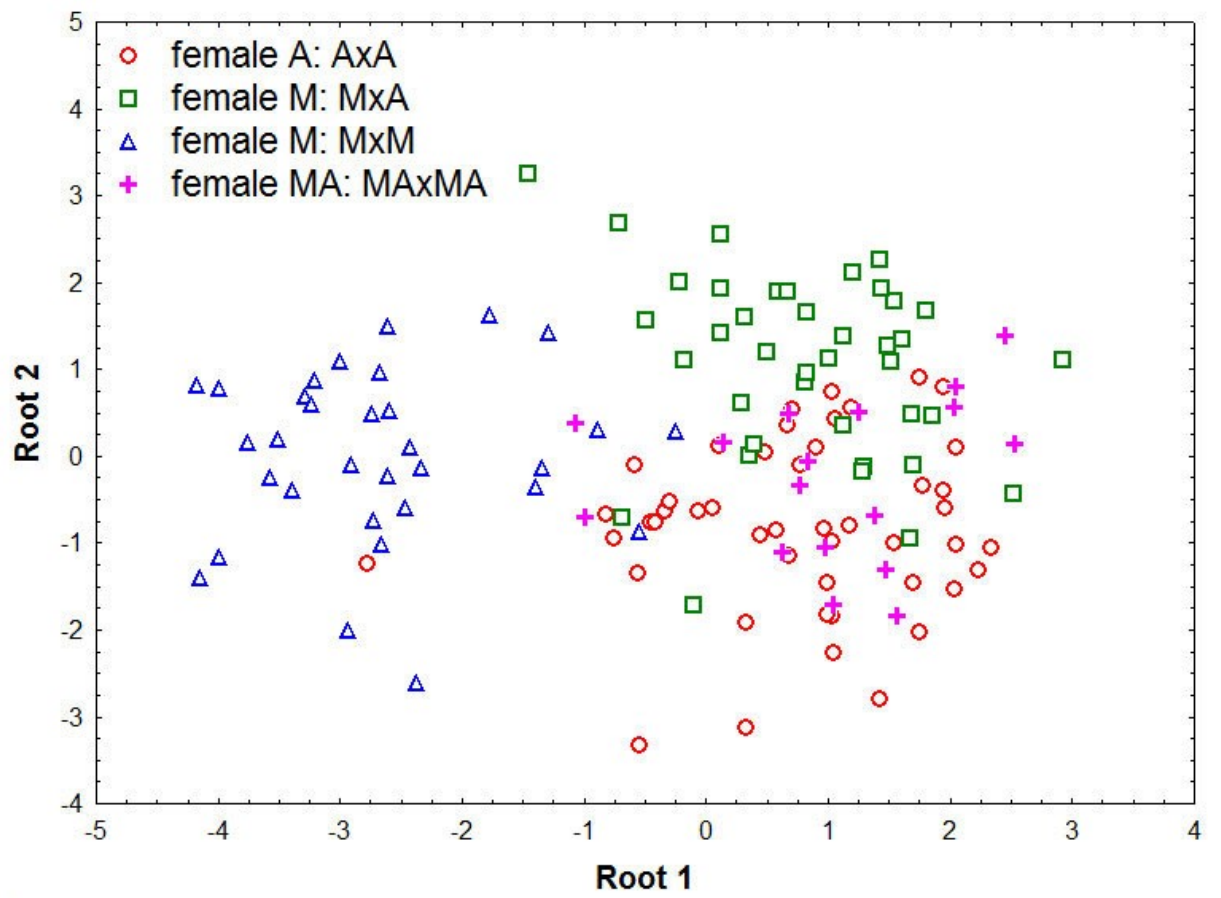


Fig. 2.

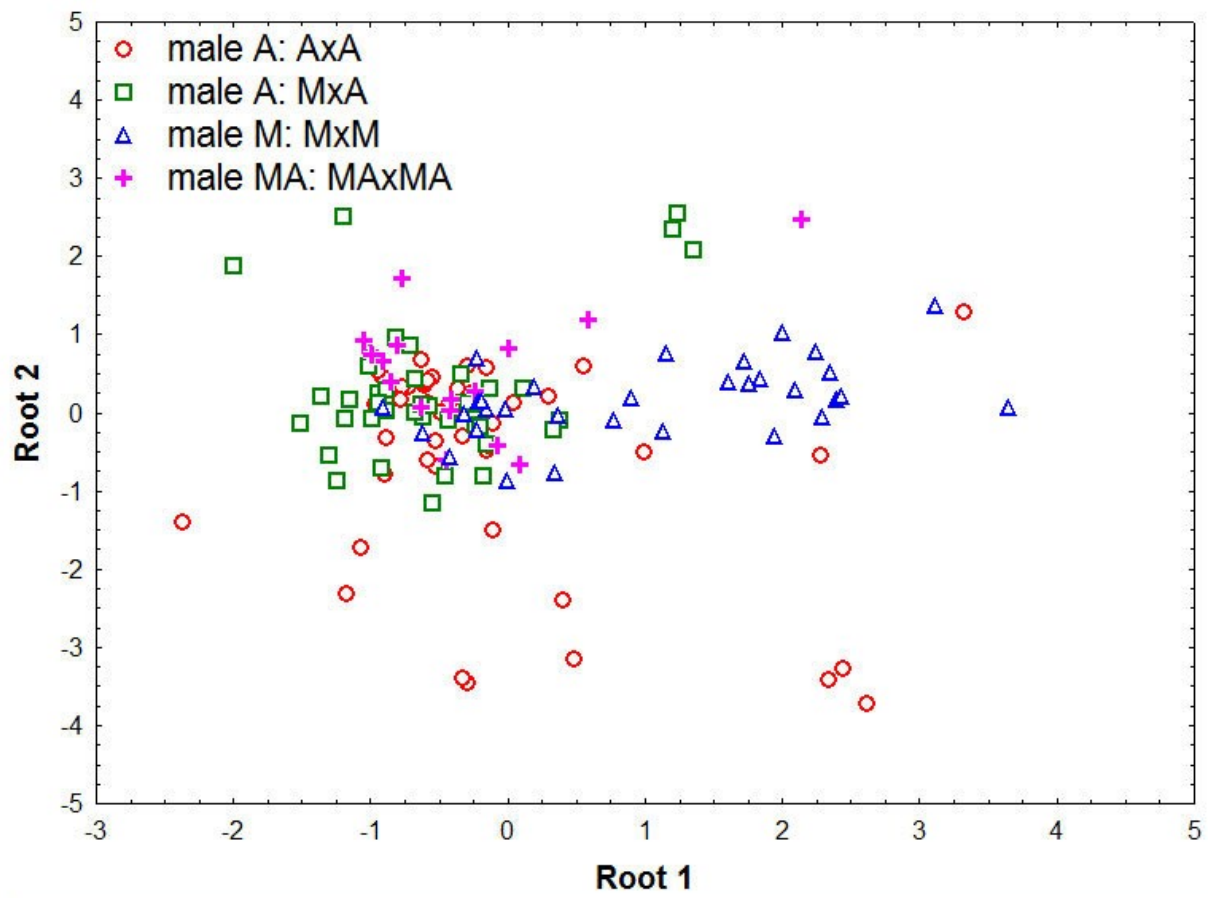


Fig. 3.

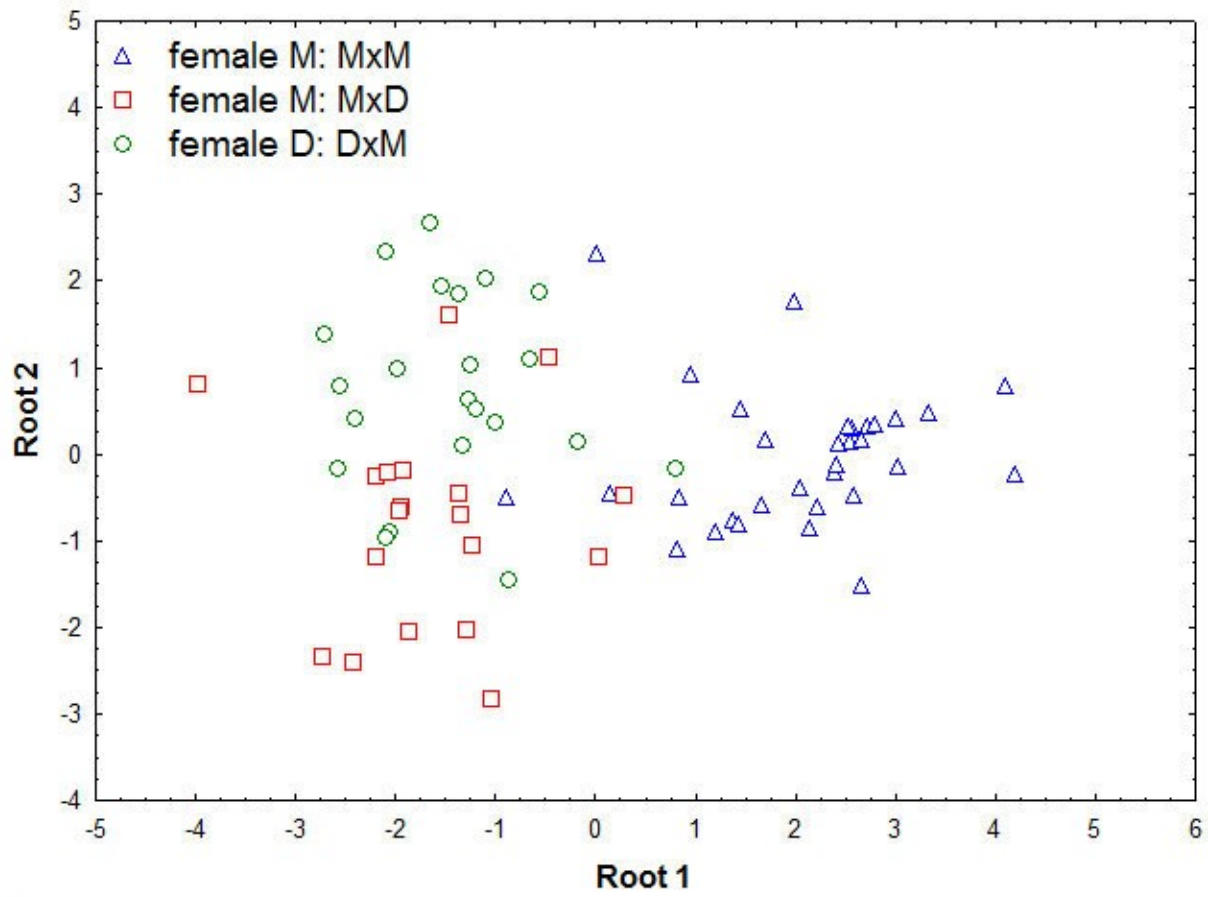


Fig. 4.

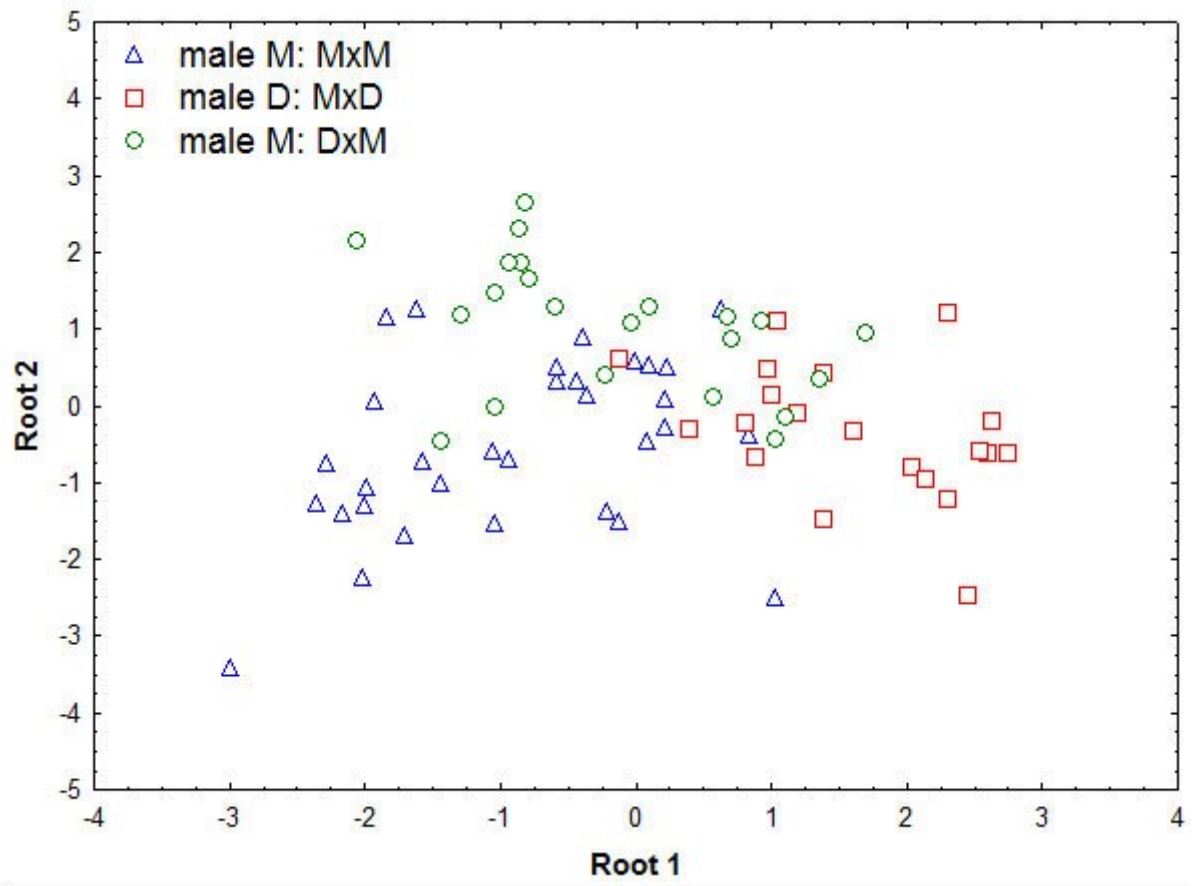


Fig. 5.

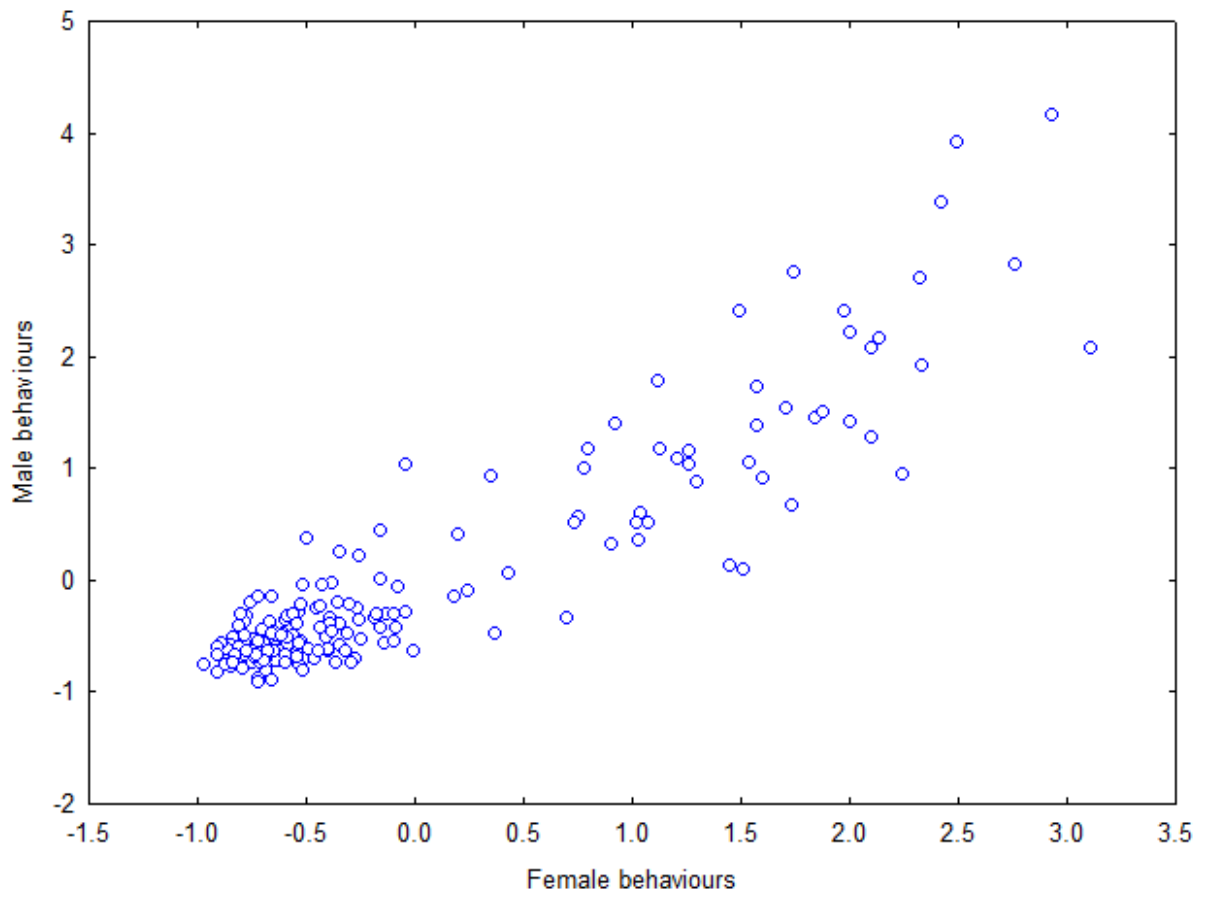
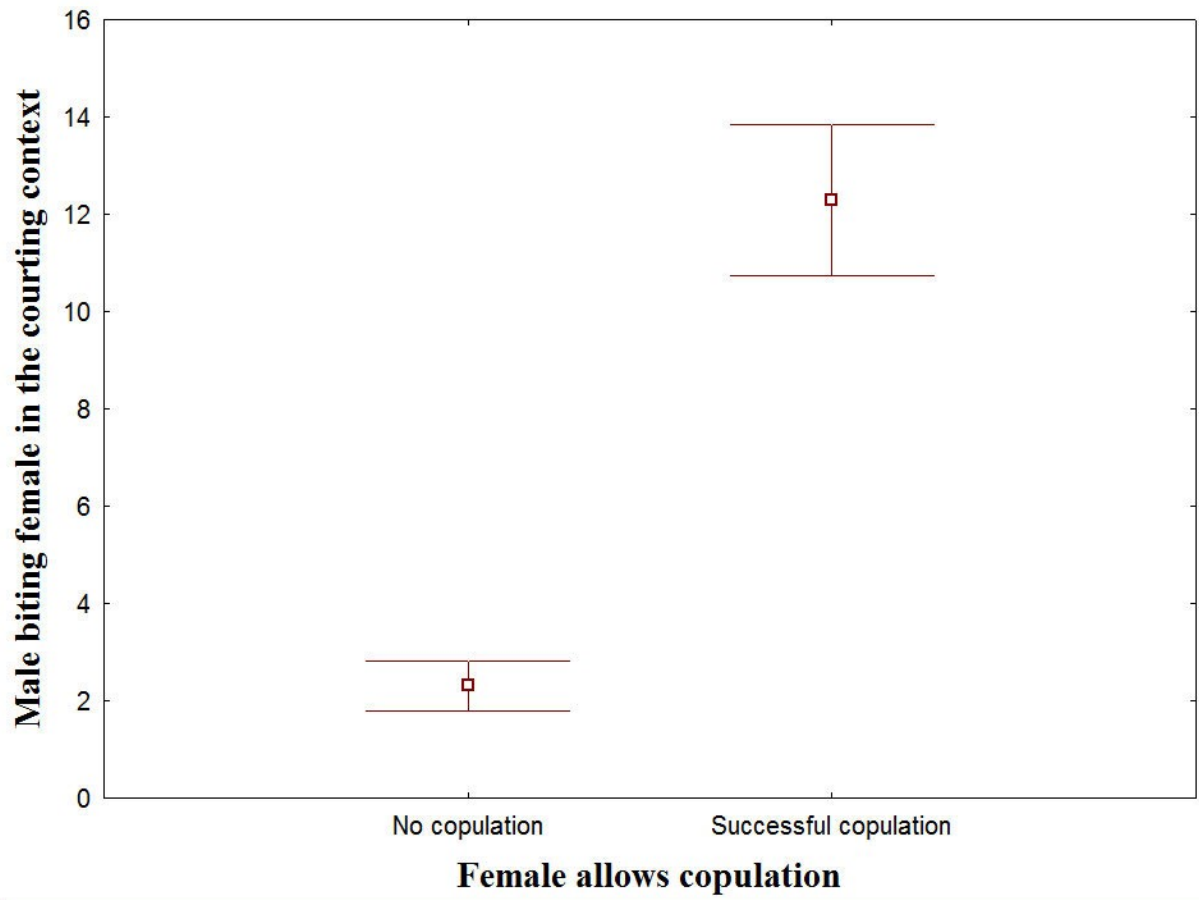


Fig. 6.



Tab.1.

Variables names and category	Variables measured as	Transformation	Kruskal-Wallis and Tukeys post-hock test	DFA CVA	CA	Multiple regression	GLM model for binary data
Male mating behaviours							
Male attack	Binary data (Yes/No)	NO			X		
Frequency of tongue flicking	Frequency	Square-root *	X	X	X		independent variable
Latency of tongue flicking	Latency	LN	X	X			
Latency of approaching	Latency	LN	X	X	X		
Latency of vibration	Latency	LN	X	X	X		
Latency of biting in sexual context	Latency	LN	X	X	X		
Latency of ejaculation	Latency	LN	X	X			
Duration of unconcern	Duration	Arcsine, Square-root **	X	X	X		independent variable
Duration of approaching	Duration	Arcsine, Square-root **	X	X	X		independent variable
Duration of freezing	Duration	Arcsine, Square-root **	X	X	X		independent variable
Duration of vibration	Duration	Square-root	X	X	X		
Duration of biting in sexual context	Duration	Square-root	X	X	X		independent variable
Duration of ejaculation	Duration	Square-root	X	X			
Duration of copulation attempts	Duration	Arcsine, Square-root **	X		X		
Biting the female in sexual context	Frequency	Square-root *	X				independent variable

Female mating behaviours						
Frequency of tongue flicking	Frequency	Square-root *	X	X	X	
Latency of tongue flicking	Latency	LN	X	X		
Latency of approaching	Latency	LN	X	X	X	
Latency of refusing	Latency	LN	X	X	X	
Latency to allow copulation	Latency	LN	X	X		
Duration of female unconcern	Duration	Arcsine, Square-root **	X	X	X	
Duration of approaching	Duration	Arcsine, Square-root **	X	X	X	
Duration of freezing	Duration	Arcsine, Square-root **	X	X	X	
Duration of refusing	Duration	Square-root	X	X	X	
Duration of allowing copulation	Duration	Square-root	X	X		dependent variable
Female biting the male	Binary data (Yes/No)	NO	X	X	X	
Allowing copulation = Male's copulation success	Binary data (Yes/No)	no, logit link function in R model	X	X	X	dependent variable

Correction for different length of experiments and data transformation used for some behavioural traits: * For the frequency data was used square-root transformation of (frequency of behaviour * (1800 / duration of experiment in seconds)), ** For duration data were used arcsin and square-root transformations of (duration of behaviour / duration of experiment in seconds).

Tab. 2.

Crossing abbreviation Female behaviours	Reclassification success [%]	AxA	MxA	MxM	MAxMA	No. of examining pairs
Female A: AxA	83	39	7	1	0	47
Female M: MxA	77	7	30	0	2	39
Female M: MxM	91	2	0	29	1	32
Female MA: MAxMA	18	8	5	1	3	17

Tab. 3.

Female mating behaviours	Root 1	Root 2	Root 3
Duration of freezing	-0,1845	-0,7447	-1,2030
Frequency of tongue flicking	-0,4110	0,5840	-0,3878
Latency to allow copulation	0,7178	0,3341	-0,1243
Latency of refusing	-0,5379	-0,1422	-0,3720
Duration of allowing copulation	1,0648	-0,8427	-0,2541
Duration of unconcern	0,7388	-0,1027	-1,5761
Duration of refusing	0,3326	-0,5716	-0,6864
Latency of approaching	-0,3238	0,5838	-0,1636
Successful copulation	-0,2366	1,0557	-0,6360
Female bites male	-0,1297	-0,1800	-0,4506

Tab. 4.

Crossing abbreviation Male behaviours	Reclassification success [%]	AxA	MxA	MxM	MAxMA	No. of examining pairs
Male A: AxA	53	25	18	4	0	47
Male A: MxA	49	17	19	3	0	39
Male M: MxM	53	13	2	17	0	32
Male MA: MAxMA	0	5	10	2	0	17

Tab. 5.

Male mating behaviours	Root 1	Root 2	Root 3
Latency of ejaculation	-0,9047	-0,3764	-0,2653
Frequency of tongue flicking	0,7144	0,0225	0,5546
Duration of un concern	0,0938	1,2329	0,7430
Duration of biting	-0,3126	0,9541	-0,6409

Tab. 6.

Crossing abbreviation Female behaviours	Reclassification success [%]	MxM	MxD	DxM	No. of examining pairs
Female M: MxM	94	30	1	1	32
Female M: MxD	80	1	16	3	20
Female D: DxM	77	1	4	17	22

Tab. 7.

Female mating behaviours	Root 1	Root 2
Duration of freezing	1,0098	0,4669
Latency to allow copulation	-0,9949	0,1802
Duration of allowing copulation	-1,1395	-0,4386
Duration of refusing	-0,1623	1,0542
Latency of refusing	0,3871	0,4947
Duration of approaching	0,2578	0,5607

Tab. 8.

Crossing abbreviation Male behaviours	Reclassification success [%]	MxM	MxD	DxM	No. of examining pairs
Male M: MxM	72	23	2	7	32
Male D: MxD	85	1	17	2	20
Male M: DxM	73	2	4	16	22

Tab. 9.

Male mating behaviours	Root 1	Root 2
Duration of freezing	-0,5909	0,0177
Duration of unconcern	1,0695	0,7640
Duration of ejaculation	0,8731	-0,5191
Latency of vibration	-0,2883	0,9607
Frequency of tongue flicking	-0,2100	0,5990
Duration of vibration	0,4135	0,2924
Latency of tongue flicking	0,2196	-0,3731
Latency of ejaculation	0,4514	-0,2866
Duration of biting the female in sexual context	0,4403	0,4453

Tab. 10.

Crossing abbreviation	MxM	DxD	DxM	MxD	MDxMD	MxMD
Mother	M	D	D	M	F ₁	M
Father	M	D	M	D	F ₁	F ₁
Egg/hatching	M	D	F ₁	F ₁	F ₂	B
No. of mothers	16	10	8	7	2	6
No. of clutches	47	54	29	23	15	17
No. of eggs	90	119	56	43	29	33
Temperature [°C]	28	28	28	28	28	28
No. of incubated eggs	87	113	51	43	29	33
No. of juveniles	80	77	3	26	20	31
Egg hatchability (%)	92	68	6	60	69	94
Survived to one year	67	52	2	17	no data	no data
Survival rate [%]	84	68	67	65	no data	no data

Tab.11.

Female/Male behaviour	Tongue flicking (F)	Male attack the female (yes/no)	Approaching female (L)	Tail vibration (L)	Biting (L)	Unconcern about female (D)	Approaching female (D)	Freezing (D)	Tail vibration (D)	Biting female (D)	Attempt copulation (D)
Tongue flicking (F)	0,0839	-0,2991	-0,1305	-0,1884	0,0158	0,0474	-0,1797	-0,0874	-0,0027	0,0688	0,2157
Female biting the male (yes/no)	0,1258	0,3390	-0,0807	-0,1195	-0,2531	-0,0437	0,0410	0,1901	0,2374	-0,0251	-0,2146
Copulation (yes/no)	0,1986	-0,1373	-0,1212	-0,3237	-0,4294	-0,5288	-0,0643	-0,0590	0,2864	0,7652	0,7484
Approaching male (L)	0,0292	0,1192	0,2515	0,1604	0,0427	0,0248	0,0901	0,0247	-0,0277	-0,0512	-0,0676
Refusing (L)	-0,0612	-0,2023	0,2348	0,1306	0,1183	-0,0205	-0,0867	-0,2246	-0,1184	0,1934	0,3068
Unconcern about male (D)	-0,5135	-0,0166	0,2293	0,4182	0,4195	0,7101	-0,1526	-0,2993	-0,4944	-0,6638	-0,4988
Approaching male (D)	-0,0573	-0,3261	-0,0929	-0,1272	-0,0422	-0,0716	-0,0015	-0,1846	0,0105	0,1988	0,2480
Freezing (D)	0,4193	0,0051	-0,1460	-0,3254	-0,2044	-0,4654	-0,1129	0,4370	0,3424	0,3848	0,3136
Refusing (D)	0,2436	0,3664	-0,0641	-0,0141	-0,1797	-0,2354	0,3621	0,2881	0,2493	-0,0239	-0,2752

Tab. 12.

Crossing abbreviation	Estimate	SE	z value	P	
Intercept	27.1154	13.3360	2.033	0.0420	*
MxA	-3.6351	3.9303	-0.925	0.3550	
MAxMA	4.5857	2.9916	1.533	0.1253	
MxM	5.3724	3.0303	1.773	0.0762	
MxD	3.1749	3.4652	0.916	0.3596	
DxM	3.0236	3.0114	1.004	0.3153	
Unconcern (D)	-20.2186	8.3467	-2.422	0.0154	*
Approaching (D)	-32.3385	14.4953	-2.231	0.0257	*
Freezing (D)	-23.8151	11.3990	-2.089	0.0367	*
Vibration (D)	0.5755	0.3872	1.487	0.1371	
Biting (D)	0.5167	0.2237	2.310	0.0209	*

Supplement 1.

Female	N	Tongue flicking			Approaching to male			Freezing	
		Reacting females (%)	Mean latency (sec)±SE	Mean frequencies ±SE	Reacting females (%)	Mean latency (sec)±SE	Mean duration (sec)±SE	Reacting females (%)	Mean duration (sec)±SE
Female A: AxA	47	70	123,4 ± 37,6	8,4 ± 1,7	81	100,9 ± 37,5	102,6 ± 15,3	83	398,5 ± 34,7
Female M: MxM	32	97	108,9 ± 26,2	25,4 ± 3,0	97	68,0 ± 14,4	199,4 ± 15,8	100	371,7 ± 40,5
Female M: MxA	39	95	231,7 ± 50,1	16,1 ± 2,9	97	179,7 ± 41,8	157,6 ± 23,6	67	176,1 ± 46,8
Female MA: MAxMA	17	82	245,4 ± 120,9	11,4 ± 2,3	94	197,2 ± 82,8	58,3 ± 12,3	100	260,9 ± 57,7
Female M: MxD	20	95	222,1 ± 79,2	15,9 ± 3,1	95	147,9 ± 63,4	201,6 ± 43,3	55	355,0 ± 69,6
Female D: DxM	22	100	123,8 ± 40,8	21,3 ± 3,6	100	78,5 ± 36,8	166,1 ± 22,8	82	259,4 ± 27,9
Total Sum	177								

Refusing of a male			Female biting the male		Allowing of a copulation		
Reacting females (%)	Mean latency (sec)±SE	Mean duration (sec)±SE	Reacting females (%)	Mean frequencies ±SE	Reacting females (%)	Mean latency (sec)±SE	Mean duration (sec)±SE
94	147,7 ± 30,5	175,5 ± 32,8	49	2,0 ± 0,3	15	278,3 ± 56,8	170,2 ± 78,9
34	337,6 ± 118,0	18,7 ± 6,7	16	1,2 ± 0,2	50	317,2 ± 34,6	75,9 ± 8,8
87	294,8 ± 64,2	60,7 ± 13,0	18	1,4 ± 0,4	13	462,8 ± 101,4	43,2 ± 7,3
94	209,8 ± 73,2	128,5 ± 31,8	53	1,2 ± 0,2	18	808,9 ± 412,8	89,2 ± 37,5
60	387,1 ± 136,8	14,6 ± 5,0	15	2,3 ± 0,9	40	438,2 ± 158,5	174,8 ± 83,2
82	323,5 ± 78,5	36,2 ± 6,0	50	2,8 ± 0,5	14	489,2 ± 185,4	72,2 ± 28,0

Supplement 2. The frequencies, latencies and duration time of the male behavioural traits (mean ± SE are given).

Male	N	Tongue flicking			Approaching to the female			Freezing	
		Reacting males (%)	Mean latency (sec) ±SE	Mean frequencies ±SE	Reacting males (%)	Mean latency (sec) ±SE	Mean duration (sec) ±SE	Reacting males (%)	Mean duration (sec) ±SE
Male A: AxA	47	98	99,0 ± 17,9	28,9 ± 3,4	100	76,7 ± 16,9	231,3 ± 30,9	96	236,6 ± 49,3
Male M: MxM	32	100	52,1 ± 9,8	55,4 ± 7,3	100	33,2 ± 6,9	239,6 ± 16,8	97	196,4 ± 17,9
Male A: MxA	39	100	114,4 ± 29,8	24,7 ± 3,0	100	113,1 ± 30,4	209,1 ± 25,8	79	365,6 ± 33,8
Male MA: MAxMA	17	100	55,8 ± 14,9	24,6 ± 3,0	100	32,0 ± 10,4	184,2 ± 25,0	100	135,0 ± 26,2
Male D: MxD	20	95	77,4 ± 26,0	37,9 ± 6,7	100	72,8 ± 26,5	201,3 ± 25,0	50	94,0 ± 22,1
Male M: DxM	22	100	80,5 ± 38,5	58,4 ± 8,4	100	77,5 ± 38,3	204,5 ± 19,5	86	182,4 ± 22,0
Total Sum	177								

Tail vibration			Male biting the female			Copulation (with ejaculation)			Attack
Reacting males (%)	Mean latency (sec) ±SE	Mean duration (sec) ±SE	Reacting males (%)	Mean latency (sec) ±SE	Mean duration (sec) ±SE	Successful copulation (%)	Mean latency (sec) ±SE	Mean duration (sec) ±SE	Male attack on female (%)
79	242,5 ± 57,7	18,1 ± 3,0	64	337,5 ± 75,3	43,6 ± 14,1	6	273,2 ± 97,6	38,1 ± 13,0	23
94	129,0 ± 36,0	12,0 ± 1,4	69	126,2 ± 37,3	97,6 ± 15,8	50	376,5 ± 45,3	23,8 ± 3,0	6
69	234,7 ± 62,1	18,6 ± 2,5	77	265,1 ± 49,1	80,4 ± 29,0	13	719,3 ± 276,0	12,4 ± 3,2	13
65	194,0 ± 74,1	16,5 ± 3,9	76	373,4 ± 130,7	28,9 ± 8,7	18	877,8 ± 449,9	20,2 ± 5,5	29
80	121,2 ± 52,6	17,5 ± 3,6	60	184,5 ± 109,3	139,3 ± 38,9	40	753,4 ± 202,8	52,3 ± 19,4	0
68	294,9 ± 96,8	19,4 ± 3,1	45	263,3 ± 76,5	63,4 ± 23,3	14	582,0 ± 197,0	13,0 ± 2,4	9

III.

Experimental crossing of two distinct species of leopard geckos, *Eublepharis angramainyu* and *E. macularius*: viability, fertility and phenotypic variation of the hybrids

Jitka Jančúchová-Lásková, Eva Landová, Daniel Frynta

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RESEARCH ARTICLE

Experimental Crossing of Two Distinct Species of Leopard Geckos, *Eublepharis angramainyu* and *E. macularius*: Viability, Fertility and Phenotypic Variation of the Hybrids

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Data Availability Statement: All relevant data are within the paper.

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Abstract

Hybridization between distinct species of animals and subsequent genetic introgression plays a considerable role in the speciation process and the emergence of adaptive characters. Fitness of between-species hybrids usually sharply decreases with the divergence time of the concerned species and the divergence depth, which still allows for a successful crossing differs among principal clades of vertebrates. Recently, a review of hybridization events among distinct lizard species revealed that lizards belong to vertebrates with a highly developed ability to hybridize. In spite of this, reliable reports of experimental hybridizations between genetically fairly divergent species are only exceptional. Here, we show the results of the crossing of two distinct allopatric species of eyelid geckos possessing temperature sex determination and lacking sex chromosomes: *Eublepharis macularius* distributed in Pakistan/Afghanistan area and *E. angramainyu*, which inhabits Mesopotamia and adjacent areas. We demonstrated that F₁ hybrids were viable and fertile, and the introgression of *E. angramainyu* genes into the *E. macularius* genome can be enabled via a backcrossing. The examined hybrids (except those of the F₂ generation) displayed neither malformations nor a reduced survival. Analyses of morphometric and coloration traits confirmed phenotypic distinctness of both parental species and their F₁ hybrids. These findings contrast with long-term geographic and an evolutionary separation of the studied species. Thus, the occurrence of fertile hybrids of comparably divergent species, such as *E. angramainyu* and *E. macularius*, may also be expected in other taxa of squamates. This would violate the current estimates of species diversity in lizards.

Introduction

The fact that related species of animals are sometimes able to hybridize is known since the beginning of evolutionary biology [1]. Nevertheless, the crucial importance of hybridization of

animal species for evolutionary processes has been largely overlooked for decades (but see [2–6]). In recent years, molecular markers allowed zoologists to detect occurrence of natural between-species hybrids in the field. As a result, presence of hybrid zones and/or introgressed genes has been documented in many animal taxa (e.g., fruit flies: [7]; butterflies: [8]; fishes: [9]; toads: [10]; snakes: [11]; lizards: [12]; Darwin's finches: [13]; nightingales: [14]; house mice: [15]; dolphins: [16]). This suggests that at least in the terminal branches of the phylogenetic tree, a predominantly divergent pattern of evolution caused by cladogenesis may be supplemented by a complementary process (syngensis). This process breaks incomplete reproductive isolation mechanisms (RIMs) among related species, enabling genetic introgression from a donor species to a recipient one. The recipient populations may benefit from a gene flow supplying alien alleles. These effects on the fitness have been already tried and tested in the donor population. A recombination with the introgressed alleles can give rise to hopeful transgressive phenotypes with extreme trait values exceeding the combined range of parental species [4, 17–19]. Moreover to these evolutionary advantages, especially hybrids of the first filial generation and backcrosses, may improve their fitness due to overdominance and/or masking of the deleterious recessives, usually referred to as heterosis or hybrid vigour [20–24]. In extreme cases as, e.g., some of the Darwin's finches, interspecific hybrids exhibit elevated fitness when compared with the parental species and genetic identities of the species have become fuzzy [25].

Hybrid sterility and/or inviability contribute fundamentally to reproductive isolation and delimitation of animal species. In a typical case, fitness of between-species and sometimes also between-population hybrids, especially those of F_2 and other segregating generations, is considerably reduced. This phenomenon is referred to as an outbreeding depression [20, 26]. Dobzhansky (1936, 1937) [27, 28] and Muller (1940, 1942) [29, 30] recognized that the easiest way to the evolution of postzygotic reproductive isolation mechanisms (RIMs) of this kind is a genetic interaction (incompatibility) of alleles belonging to separate genes (loci). The original prevailing $A_1A_1B_1B_1$ genotype is replaced with $A_2A_2B_2B_2$ in the daughter population that becomes reproductively isolated due to reduced fitness of the hybrids (typically $A_1A_1B_2B_2$ and $A_2A_2B_1B_1$). Accumulation of Dobzhansky-Muller incompatibilities (DMIs) is probably a function of time that elapsed from the divergence of the crossed species [31]. This theoretical prediction was corroborated by experimental data in multiple taxa of animals (e.g., in frogs [32], in pigeons and doves [33], in centrarchid fishes [34], in *Drosophila* fruit fly [35, 36], in galliform birds [37], *Triturus* newt [38], but see [39] for the main role of sexual selection in hybridizing sword tail fishes). However, little is known about evolutionary rate at which these incompatibilities arise.

In vertebrates, hybrids of extremely distant genera were reported in fishes (e.g., Lepisosteidae: *Lepisosteus* and *Atractosteus* separated for 33–100 million years [40, 41]; Centrarchidae: *Acantharchus* and *Micropterus* separated for ~35 million years [34] and frogs (e.g., Hylidae: *Hyla* and *Pseudacris* separated for 22–80 million years [42, 43]). The time required for accumulation of efficient postzygotic RIMs varies considerably even among the principal clades of amniots (for details of genetic divergence in lizards, see the review [44]). The best documented comparison represents at least five-fold difference between mammals, typically losing the ability to produce viable F_1 hybrids after one or two million years of separation, and birds losing this ability after 20 million years [45–47]. Divergence time estimates reported for marine turtles producing viable hybrids are even longer (e.g., *Chelonia* x *Caretta* [42], estimated to ~63 mye [48]). Vital and sometimes also fertile hybrids of distinct species/genera are also known for other chelonian taxa (e.g., Bataguridae: *Cyclemys* x *Occadia* [49]; *Mauremys* x *Sacalia* [50]; Chelidae: between some of the species in the genus *Chelodina* [51]). This may be attributed to a slow mutation rate reported in the chelonians [52]. The crocodylians, a sister taxon of the birds, are also able to produce viable between-species hybrids (e.g., *Crocodylus siamensis* x *C.*

rhomboifer [53]). Nevertheless, the genus *Crocodylus* is relatively young; the oldest records of this genus are known from the end of the Miocene [54]. In contrast to the high level of species diversity of lizards and snakes, there is only limited information about the time required to establish the postzygotic RIMs in most lineages of squamates. Most examples of viable F₁ hybrids of squamates come from unisexual species (e.g., *Leiolepis* [55]; *Darevskia* [56]; *Aspidoscelis* [57]; *Lepidodactylus* [58]; *Hemidactylus* [59]; *Heteronotia* [60]; *Nactus* [61]). In these cases, however, further reproduction of the hybrids that may be otherwise sterile is enabled by parthenogenesis and/or multiplication of the gene dosage (triploidy, tetraploidy). Except for the parthenogens and their close relatives, also viable F₁ hybrids of lizards belonging to distinct species or genera were reported in, e.g., true iguanids (e.g., *Conolophus* x *Amblyrhynchus* [62, 63]; *Ctenosaura similis* x *C. bakeri* [64]; *Iguana iguana* x *I. delicatissima* [65]) and lacertids (e.g., within the genus *Lacerta*: [66–70]; within the genus *Podarcis*: [71, 72]). Similar cases were repeatedly reported in snakes, e.g., pythons (*Morelia* x *Liasis* [73]; *Python natalensis* x *P. molurus bivittatus* [74]), colubrids (*Pituophis catenifer sayi* x *Pantherophis vulpinus* [75]) and viperids (*Vipera nikolskii* x *V. berus* [76, 77]). In our previous paper [44], we reviewed the available records of hybridization events in lizards and found that the upper limit of the HKY distance of cyt b gene between parental species producing viable homoploid bisexual hybrids is 19%; the corresponding distance for parental species of parthenogenetic hybrids is 21%. We also found that the experimental studies reliably reporting and documenting their further reproductive success in lizards are exceptional (but see [66–69], for a review see [44]).

The above mentioned differences among the higher taxa of amniots in the time-scale required for the evolution of postzygotic RIMs may have fundamental consequences on speciation patterns, which should be considered in the conservation theory and practice. The risk of outbreeding depression should be considered in defining taxonomic and/or population genetic delimitation of the conservation units in endangered species [78, 79]. Too broad definition of these units leads to a rapid increase in the expenses as well as demographic and genetic risks of extinction associated with small population numbers [78, 80–82].

In search of a dyad of model lizard species with allopatric distribution ranges that have been separated by well-dated geological events, we focused on the Middle East region. The Iranian Plateau and Zagros Mountains represent a distinct geographic barrier that limits the distribution and prevents contacts between lowland dwellers of Mesopotamia-Persian Gulf and those of Central Asia and Indian subcontinent [83]. History of these units is precisely known according to geological evidence; they originated as a result of a collision between Arabia and Eurasia plates that started 35–20 million years ago. Nevertheless, the main uplift of this area occurred 15–12 million years ago [84, 85]. Further topography growth of the external Zagros, Alborz, Kopet Dagh and Caucasus mountain belts reached its maximum 5 million years ago [86]. The long-lasting presence of the above described geographic barrier has clear consequences on a phylogenetic and phylogeographic structure of several reptilian taxa in Iran and adjacent areas; e.g., species complexes of the *Laudakia caucasica* [87, 88], *Eremias persica* [89], and *Mesalina watsonana* [90].

Eublepharis macularius (BLYTH, 1854), a lizard belonging to the family Eublepharidae, is a common laboratory animal, which is widely used as a model species of squamate reptiles in physiological [91–95], behavioural [96, 97], and evolutionary [98, 99] research. The distribution range of *E. macularius* includes large territories of Afghanistan, Pakistan and India [100]. Other species of the genus *Eublepharis* [100] are also distributed on the Indian subcontinent (*E. hardwicki*, *E. fuscus*) and Turkmenistan (*E. turkmenicus*). Another distinct species of the genus *Eublepharis*, the *E. angramainyu* (ANDERSON AND LEVITON, 1966) inhabits Mesopotamia and SW Iran [83]. The range of the *E. angramainyu* is separated from those of the *E. macularius* and remaining species of the genus *Eublepharis* by the Iranian Plateau and Zagros

Mountains [100]. Thus, the dyad of the *E. angramainyu* and *E. macularius* represents a promising model of species that underwent a long-lasting geographical isolation.

One may argue that the seashore along the Gulf of Oman was penetrable for the geckos of the genus *Eublepharis* at least in the past. However, sequence divergences between mitochondrial genes of the *E. macularius* and *E. angramainyu* are considerable (uncorrected p-distances for 303 bp fragment of cyt b gene exceed 19%; HKY85 distance 22%, Palupčiková unpublished data) and fully congruent with the geological dates of the main uplift of the Iranian Plateau.

The aim of this paper is to examine the ability of distinct lizard species evolving separately for several million years to hybridize and exchange genes. For this purpose we crossed the *E. angramainyu* and *E. macularius* under laboratory conditions and assessed (1) viability, (2) fertility and (3) phenotypic characters (body size, body shape, coloration pattern) of the hybrids and parental species. Successful production of viable and fertile F₁ crosses of our model species would further support the hypothesis that lizards possess slow (“avian” or “chelonian”) rather than rapid (“mammalian”) pattern of postzygotic RIM acquisition [44]. In accord with the general model of Dobzhansky-Muller incompatibilities and the empirical evidence in other animal taxa [101], we predicted that putative fitness losses affect more hybrids of F₂ generation than those of F₁ generation (all possessing a genotype A₁A₂B₁B₂).

Materials and Methods

Ethics Statement

All performed experiments were allowed by institutional Animal Care and Use Committee of the Charles University in Prague, and approved by Ethical Committee of Ministry of Education, Youth and Sports of the Czech Republic license no. 18147/203 and 24773/2008–10001. All animals from nature were purchased from a Czech company importing animals in the year 2002 and from private breeders. *Eublepharis* sp. does not belong to the species whose trade is limited by the CITES agreement or any other known regulations. According to the IUCN categorization it neither belongs to endangered species. After the study, geckos were used either for other behavioural experiments or for breeding purposes.

Experimental procedures

The breeding stocks of the parental species were 38 females and ten males of an *E. macularius* (the first generation of descendants of wild-caught animals imported from Pakistan) and only five females and three males of the rare *E. angramainyu* (wild-caught animals and their two daughters; a putative locality of origin: Choqa Zambil, Khuzestan province, Iran, 32°00'N 48°31'E, for more details about the locality see [102]).

To obtain F₁ hybrids, 17 virgin females of the *E. macularius* were allowed to copulate with one breeding male of the *E. angramainyu*. The resulting F₁ hybrids were reared to sexual maturity and further bred to obtain F₂ hybrids and/or backcrosses with either *E. macularius* or with the same breeding male of the *E. angramainyu* (with their father). Fertility of some of the backcross hybrids was subsequently assessed by crossing with the parental species (for details see under the [Results](#) and [Table 1](#)). Because the geckos of the genus *Eublepharis* are able to store sperm for several months, each experimental female was allowed to copulate exclusively with a single male during a given mating season (lasting from January/February to July/August). In contrast, males were allowed to copulate with multiple females within a single breeding season. 15 F₁ hybrid females were experimentally crossed for more than one breeding season; this allowed us to test their fertility with two or three different males (first with F₁ male or one of the parental species and then with a male of the other parental species). As controls for the hybridization experiments, 16 females of the *E. macularius* and five *E. angramainyu* females

Table 1. The incubation success of eggs (hatchability) and survival rates of hatchlings. The parental species (*E. macularius*— P_M , *E. angramainyu*— P_A), their hybrids of the first (F_1) and second (F_2) filial generations, backcrosses of F_1 females to male of *E. angramainyu* (B_{1A} ; denoted as $MAxMA$), the reciprocal backcrosses of F_1 males or females to *E. macularius* (B_{1M} ; the individuals with father F_1 hybrid are denoted as $MxMA$, while those with mother F_1 hybrid as $MAxM$), and two categories of higher order hybrids (crosses of $MxMA$ females with males of either *E. macularius* or *E. angramainyu*). The above mentioned generations and/or crossings refer to the embryos and hatchlings.

Crossing abbreviation	M	A	MA	MAxMA	MAxMA	MAxM	MxMA	(MxMA)xA	(MxMA)xM
Mother	P_M	P_A	P_M	F_1	F_1	F_1	P_M	B_{1M}	B_{1M}
Father	P_M	P_A	P_A	F_1	P_A	P_M	F_1	P_A	P_M
Egg/hatchling	P_M	P_A	F_1	F_2	B_{1A}	B_{1M}	B_{1M}	$B_{1M}xP_A$	B_{2M}
No. of mothers	16	5	17	13	10	22	10	3	2
No. of clutches	47	26	37	41	24	68	29	10	4
No. of eggs	90	42	71	81	42	131	57	17	7
Temperature [°C]	28	26	28	28	26	28	26	28	28
No. of incubated eggs	87	38	70	55	16	16	15	106	18
No. of juveniles	80	13	31	4	0	0	0	44	6
Egg hatchability (%)	92	34	44	6	-	-	40	75	8
Survived to one year	67	11	28	1	0	0	39	27	1
Survival rate (%)	84	85	90	25	-	-	78	66	100
Sex ratio: Males/females	9/58	4/7	3/25	0/1	0/0	0/0	4/35	2/25	0/1

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were bred with conspecific unrelated males (with the exception of two *E. angramainyu* females, which were the daughters of the breeding male).

The animals were housed individually in glass terrariums 60 x 30 x 20cm or 30 x 30 x 20cm in size. The ambient temperature in the breeding room was about 28°C with permanent presence of basking cables under every terrarium to maintain a temperature gradient. The floor of each cage was covered with bark substrate. Paper shelters, as well as feeding and drinking dishes, were provided. During the laying season, containers with adequately humid coconut substrate for egg deposition were added. The geckos had continuous access to water and were fed crickets and mealworms dusted with vitamins and minerals (Nutri Mix) weekly; AD₃ and E vitamins were provided once per 14 days. The hatchlings were housed singly in plastic boxes 20 x 20 x 15cm and were fed solely the vitamins dusted crickets up to the three months of their age.

We studied the following nine categories of the parental species and their hybrids that are further referred to as follows (the abbreviations are given in parentheses; on the first place there is always an abbreviation for a female, then cross (x) with a male on the second position; the number and the sexes of these specimens in [Table 1](#)):

1. P_M —the parental generation of the *E. macularius*, both parents belong to the *E. macularius* (M);
2. P_A —the parental generation of the *E. angramainyu*, both parents belong to the *E. angramainyu* (A);
3. F_1 —the first generation hybrid, a mother of the *E. macularius* and a father of the *E. angramainyu* (MA);
4. F_2 —the second generation hybrid, both parents are F_1 hybrids of the *E. macularius* and *E. angramainyu* (MAxMA);
5. B_{1A} —the first generation backcross with the *E. angramainyu*, a mother is an F_1 hybrid and a father belongs to the *E. angramainyu* (MAxMA);

6. B_{1M} –the first generation backcross with the *E. macularius*, a mother is an F_1 hybrid and a father belongs to the *E. macularius* (MAxM);
7. B_{1M} –the first generation backcross with the *E. macularius* (reciprocal to 6), a mother belongs to the *E. macularius* and a father is an F_1 hybrid (MxMA);
8. $B_{1M} \times P_A$ –a higher order hybrid, a mother is the B_{1M} hybrid (cf. 7) and a father belongs to the *E. angramainyu* ((MxMA)xA);
9. B_{2M} –the second generation backcross with the *E. macularius*, a mother is the B_{1M} hybrid (cf. 7) and a father belongs to the *E. macularius* ((MxMA)xM).

Respective to the nocturnal activity pattern of the geckos and their thermal preferences [103], the mating attempts were conducted in the evening (after 7 p.m.) in a temperature-controlled breeding room (28°C) illuminated by a single red 25-W light bulb. Prior to the experiment, the females were weighed and were controlled for their receptivity by a visual inspection of the follicular growth through the abdomen wall [93]. We gently placed the male into the female's terrarium for 30 min and we recorded the copulation behaviour using a night vision video camera. If mating did not occur within this interval, we repeated the trial the other day. The primary aim was to allow successful mating and to enable the production of fertilized eggs.

During the egg-laying season (since February to September), we controlled the egg-deposition containers for three times a week. The eggs were weighted and placed to the temperature-controlling incubator in plastic boxes, each containing a single clutch. We set the temperature to $28.5 \pm 0.5^\circ\text{C}$, which is an optimal and preferred incubation temperature in the *E. macularius* [97, 104, 105]. Nevertheless, according to our previous experience with the *E. angramainyu*, the successful development of their embryos require slightly lower temperatures and longer incubation time. At 28°C incubation temperature (an upper limit for successful incubation), some hatchlings possessed a prolapsed yolk pouch. After consultation with other experienced colleagues at this field (e.g. Lukáš Kratochvíl, Charles University), we set the incubation temperature to $26 \pm 0.5^\circ\text{C}$ for the eggs laid by the *E. angramainyu*. The only feasible solution was to perform the experiments within the temperature range of 26–28°C, among which the incubation temperature overlaps in both species included in the experiment. Consequently, the eggs laid by the F_1 hybrid females were initially incubated either at 26°C or at 28°C to compare the hybrid hatchability at the optimum incubation temperature for both parent species (at 26°C in *E. angramainyu* and at 28°C in *E. macularius*). The temperature was selected at random for the first clutch and then regularly switched in successive ones (see Table 1). In additional backcrossing of the F_1 females with the *E. macularius* males in the breeding season 2013, which was aimed to prove their fertility, the incubation temperature was set to 28°C.

For every egg we took down the identity of the parents, the dates of laying and hatching, the weights of egg and hatchling and the incubation temperature. In order to perform formal tests of the hatchability, we used GLMs, in which the hatching of the incubated eggs of an individual clutch (number of hatchlings of one clutch and number of non-hatched eggs of the same clutch) was given as a dependent variable with binomial distribution and logit link function; the juvenile form, the incubation temperature and its interactions, and the clutch sequence were introduced as category explanatory variables. The calculations were performed in the R (R Development Core Team, Vienna, Austria).

Most eggs that have failed to hatch until the standard terms [106] were dissected to prove the presence and developmental stage of the embryos. Nevertheless, the content of many rotten eggs was entirely decayed, which precluded a reliable dissection. Thus, in many cases, we were unable to distinguish the fertilized eggs from those unfertilized.

The hatchlings were weighted and scanned (a ventral and a dorsal view of the body) in standardized positions. This procedure was repeated in adulthood at the age of 2–3 years. In order to provide a reference in the form additional fully grown individuals, the data set was supplemented with adult specimens of *E. macularius* from Pakistan and *E. angramainyu* from Iran (both wild-caught individuals and their descendants). In total, we collected 91 valid records for juveniles (*E. angramainyu*– 4 specimens, *E. macularius*– 32 spec., MA– 25 spec., MAxMA– 3 spec., MxMA– 11 spec., MAxM– 16 spec.) and 139 valid records for the animals older than two years (*E. angramainyu*– 10♀, 5♂, *E. macularius*– 55♀, 13♂), MA– 24♀, 3♂, MAxMA– 1♀, MxMA– 15♀, 2♂, MAxM– 7♀, 3♂, MMAxA– 1♀).

The coloration pattern analysis of the *E. angramainyu* (29 spec.), *E. macularius* (29 spec.), F₁ (28 spec.) and the B_{1M} (27 spec.) hybrids we conducted on a dorsal view of the head. For this purpose, we examined the scans of the animals older than one year with fully developed adult coloration pattern (Fig 1, also in [97]). First, the scans were set to black and white colors (converted to Grayscale mode, then to Bitmap mode by 50% Threshold method in Adobe Photoshop CS2; Adobe Systems Incorporated, USA). The total number of dark (melanistic) spots and the length of the longest continuous spot were performed by UTHSCSA Image Tool (San Antonio, Texas). The area of the largest continuous dark spot was measured in ImageJ program (National Institutes of Health, USA) (Fig 2). All measurements were calibrated using a squared paper present in each scan.

To test the effect of species/hybrid category on the adult coloration pattern on the head, we analyzed the Number of spots (square-root transformed), Spot size (area of the largest spot scaled to the head size and natural log-transformed) and Spot length (length of the largest spot scaled to the head length and natural log-transformed) using linear models with the form of the animal (P_M, P_A, F₁, B_{1M}) as a factor. Post hoc Tukey tests were adopted to compare the factor levels. The calculations were performed using STATISTICA, version 6.0 (StatSoft Inc., Tulsa, USA).

For morphometric analyses we adopted and/or modified standard measurements from Kratochvíl et al. (2003) [107] and Frýdlová et al. (2011) [108]. We used the following 14 measurements that were measured by UTHSCSA Image Tool from digital images: (1) SVL–snout-vent length; (2) DEX1 –distance between the extremities (from the posterior margin of the front leg to the cloacal lips); (3) DEX2 –from the posterior margin of collar to the cloacal lips; (4) TW–tail width (the largest width of the tail); (5) UFL–upper fore-limb length; (6) CFW–chest and upper fore-limb width; (7) LFL–lower fore-limb length (without hand); (8) FL–middle finger length without the claw; (9) HHW–hip upper hind-limb width; (10) KHL–knee to heel length; (11) HL–head length (from rostrum to the posterior margin of collar); (12) HW–head width, the largest width of the head; (13) EEL–distance between anterior corners of eyes; (14) REL–rostrum to eye length, from tip of the snout to the anterior corner of eye. In case of juveniles we measured only SVL. For the definition of these measurements, see Fig 2.

In order to separate a shape component of the morphometric variation, we performed the size-adjustment of the original variables. For this purpose, we used the method published by Somers (1986, 1989) [109, 110] as implemented in the Size analysis v02 [111–113]. This software computes not only generalized (multivariate) isometric size of the original untransformed measurements, but also partial isometric size-adjusted measurements. These size-free data were further analyzed by a multivariate exploratory statistics as implemented in the discriminant function analysis (DFA) subroutine of STATISTICA, version 6.0. The data were checked for normality prior to the statistical analyses. Deviations from normality were small, and most distributions were both unimodal and symmetrical as required for the used multivariate procedures.

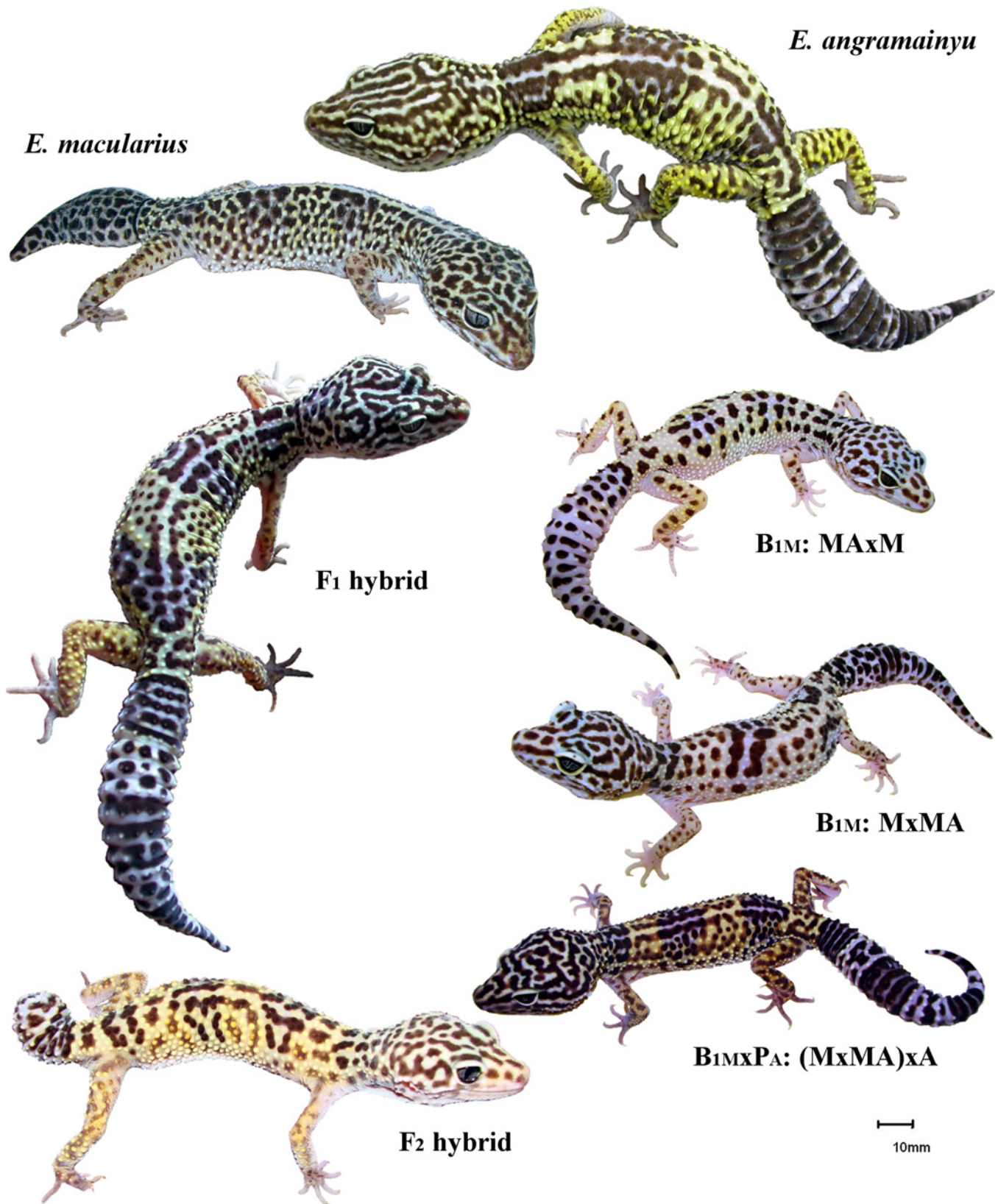


Fig 1. The external appearance and coloration. *E. macularius* (P_M), *E. angramainyu* (P_A), their hybrid of the first (F_1) and second filial generations (F_2), backcrosses of the F_1 with male or female *E. macularius* (B_{1M} : $MA \times M$ and B_{1F} : $M \times MA$, respectively), and a cross between a female of the latter backcross and a male of the *E. angramainyu* ($B_{1M} \times P_A$). The scale bar used was 10mm.

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Results

Mating success, fertility, hatching success and survival of hybrids

During five breeding seasons, the breeding male of the *E. angramainyu* was successively paired with 17 virgin females of the *E. macularius*. 15 of these females subsequently produced eggs. Since at least one egg of each female hatched, all these females were successfully fertilized by heterospecific matings. The hatchability of the F_1 hybrids was 44% ($n = 70$ incubated eggs at 28°C); this value resembles that of the *E. angramainyu* (34%, $n = 38$, 26°C), but is still apparently lower than in *E. macularius* (92%, $n = 87$, 28°C). 25 females and 3 males of 31 F_1 hatchlings survived to the age of one year (90%). The survival rate was similar to those recorded in the parental species (*E. macularius* 84%, *E. angramainyu* 85%, $n = 80$ and 13, respectively). These F_1 hybrids were further bred to obtain F_2 and/or B_1 generations (for hatching success, survival and other details of hybridization experiments, see [Table 1](#)).

In order to obtain F_2 hybrids, three F_1 hybrid males were consecutively paired with 13 F_1 hybrid virgin females (six, five and two females with respective males). Each of these 13 females copulated and laid eggs. We incubated 71 eggs (16 eggs from 12 clutches at 26°C and 55 eggs from 29 clutches at 28°C), nevertheless, only four eggs from three different F_1 hybrid females hatched. All these F_2 hybrid hatchlings were sired by a single male and incubated at 28°C (hatchability = 6%; no significant effect of temperature on hatchability was detected by Fisher exact test: $P = 0.5680$). Only one F_2 hybrid hatchling, a female, survived to the age of one year ([Fig 1](#), see its inborn malformation of the tail). None of the 18 eggs (nine clutches from five females) that were subsequently examined contained a macroscopically visible embryo.

The other 11 F_1 hybrid virgin females, as well as the six F_1 females that failed to produce F_2 or B_1 hybrids in the previous breeding season were backcrossed with males of the *E. angramainyu* or *E. macularius*. Ten of them (six virgins) were allowed to copulate with the breeding male of the *E. angramainyu*, fertility of which was proved by previous breeding records. Each female laid one egg at least. As in the case of F_2 hybrids, the eggs were incubated either at 26°C (15 eggs of 10 clutches) or 28°C (16 eggs of 14 clutches). Nevertheless, no juveniles hatched. Moreover, 15 of these eggs (nine clutches from six females) were later dissected and none of them contained a macroscopically visible embryo.

Six of the seven F_1 hybrid females (five virgin) that copulated with three males of the *E. macularius* (three, two and two females, respectively; fertility of these males was proved by previous breeding records) laid eggs and at least five of them were fertile (83%, four of them produced viable offspring, while the remaining fertile female produced just fully developed embryos that failed to hatch). The incubation temperature was randomly set either to 26°C (18 eggs from 10 clutches) or 28°C (17 eggs from 9 clutches) and then regularly switched in successive clutches of the female. In a sharp contrast with the negative results of the reverse backcrossing with *E. angramainyu* described above, 15 of these 36 eggs hatched (43% hatchability; six hatchlings at 26°C and nine ones at 28°C, no significant effect of temperature on hatchability was detected by the Fisher exact test: $P = 0.3145$). Three males and eight females survived to the age of one year (73% survival). Additional four dead embryos that failed to hatch (all from 26°C) were found inside 16 dissected eggs belonging to ten clutches produced by five F_1 hybrid females.

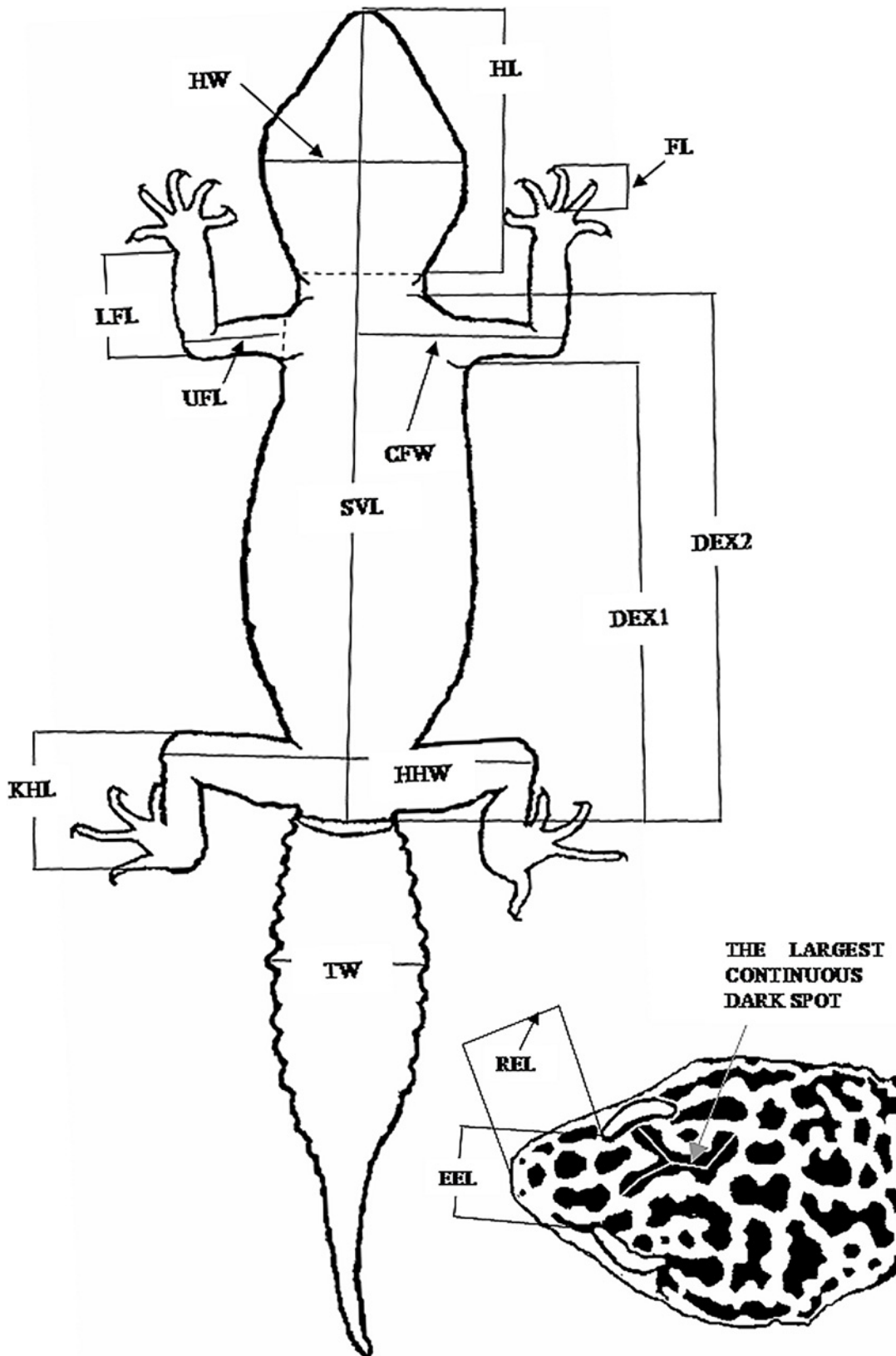


Fig 2. Measurements of the body and the head. SVL: snout-vent length; DEX1: from the margin of the front leg to the cloacal lips; DEX2: from the margin of collar to the cloacal lips; TW: tail width; UFL: upper fore-limb length; CFW: chest and upper fore-limb width; LFL: lower fore-limb length; FL: finger length; HHW: hip upper hind-limb width; KHL: knee to heel length; HL: head length; HW: head width; EEL: length between eyes; REL: rostrum to eye length; the largest spot: length and area was measured; the number of spots was computed.

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To prove their fertility, 17 F₁ females that failed to produce F₂ or B₁ hybrids in the previous experiments with effect of the incubation temperature were backcrossed again with males of the *E. macularius* in the breeding season in 2013. The eggs were incubated at 28°C only. Each female laid at least one egg and 12 of them appeared fertile (71%). Out of the 89 eggs belonging to the 49 clutches, 35 juveniles successfully hatched (hatchability = 39%). 28 of them (80%) survived up to the age of 12 months (one male and 27 females). Taken together with the above data, 16 of the 24 F₁ hybrid females (67%) were unambiguously fertile.

Ten females of the *E. macularius* were allowed to copulate with one of three F₁ hybrid males (five, two and three females with respective males). Nine of these females produced eggs, 55 eggs were incubated at 28°C and 41 juveniles hatched successfully (75% hatchability); 27 hatchlings (two males and 25 females) survived to adulthood (66% survival).

In order to test the fertility of the B₁ hybrids, three females MxMA were crossed with a male *E. angramainyu*. They produced 17 eggs; 13 eggs were incubated at 28°C and only one juvenile hatched (8%) and survived to the age of one year. Another two females MxMA were crossed with *E. macularius* males and they laid seven eggs, six of which were incubated at 28°C and one egg failed. Half of the eggs hatched but only one juvenile survived to adulthood.

To compare the incubation success (hatchability) in paternal species and the available categories of hybrids, we adopted a marginal model (geeglm function, family = binomial, logit link) accounting for an identity of the mother. The model revealed a significant variation of the incubation success among the examined groups (species and categories of hybrids; df = 8, $\chi^2 = 76.2$, $P < 0.0001$; Table 2). The incubation success of the *E. macularius* was significantly higher than those found in every other examined groups.

Most of the hatchlings successfully survived up to the age of one year; 84% of *E. macularius* (67 of 80), 85% of *E. angramainyu* (11 of 13), 90% of F₁ hybrids (28 of 31) and 72% of pooled categories of F₂, B₁ and higher order hybrids (68 of 95). The variation in the survival rate among these groups approached significance (glm, binomial response variable, logit link, $\chi^2 = 7.2$, df = 3,218, $P = 0.0666$).

Table 2. The effects of hybridization on the incubation success (hatchability) of the *E. macularius*, *E. angramainyu*, and their hybrids. Hybridization crossing - factor group; hatchability - binomial response variable comparing hatched and failed eggs of each clutch. Coefficients (Estimate), its Standard errors (SE), Wald statistics (Wald) and significance of treatment contrasts against reference group *E. macularius* (P) are provided. The marginal model (geeglm function, logit link) accounts for a mother's identity to avoid the problem of pseudoreplications. See Table 1 for explanations of the Generation and Crossing abbreviations.

Generation	Crossing abbreviation	Estimate	SE	Wald	P
	Intercept	2.4178	0.5614	18.55	< 0.0001
P _A	A	-3.0041	0.7237	17.23	< 0.0001
F ₁	MA	-2.6733	0.6359	17.67	< 0.0001
B _{1M}	MxMA	0.4566	1.1785	0.15	0.6984
B _{1A}	MAxA	-5.7987	1.0526	30.35	< 0.0001
B _{1M}	MAxM	-2.732	0.8005	11.65	0.0006
F ₂	MAxMA	-5.1546	0.8501	36.76	< 0.0001
B _{1M} xP _A	(MxMA)xA	-4.9868	0.8518	34.27	< 0.0001
B _{2M}	(MxMA)xM	-2.4178	0.5614	18.55	< 0.0001

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Morphology of the hybrids

The parental species as well as the F₁ hybrids exhibit distinct features of a physical appearance including the coloration pattern, body size and shape (for details, see Fig 1). We further examine these traits separately.

Coloration pattern. The typical patterns of dark spots on the head of adult individuals differ markedly between the *E. angramainyu* and *E. macularius*. Large elongated longitudinal spots prevail in the former species, while the presence of numerous, but smaller and rounded spots in the latter one. We examined the number of dark spots as well as the size of the largest one in both parental species and their F₁ and B_{1M} hybrids (Table 3). ANOVAs revealed a highly significant variation among these groups in both these traits (F_{3, 109} = 38.4, P < 0.0001 and F_{3, 107} = 28.9, P < 0.0001, respectively). The mean values for hybrids were between those of the parental species; F₁ hybrids were closer to the *E. angramainyu* in this respect; the B_{1M} hybrids exhibited values closer to those of the *E. macularius* (Fig 3).

Body size. Body size of the *E. angramainyu* is considerably larger than in the *E. macularius* and this difference is demonstrable both in adults and hatchlings (Figs 4 and 5). Consequently, ANOVAs revealed that the snout-vent length (SVL) varied significantly among of the examined species and their hybrids (F_{4, 132} = 44.05 and F_{5, 97} = 14.42 for adults and hatchlings, respectively; both P < 0.0001). Post hoc tests distinguished two homogenous groups (at α = 0.05; Ps of all significant comparisons are < 0.0001) according to the adult body size; the one containing the *E. angramainyu* and F₁ hybrids, and the other one consisting of the *E. macularius* and their B_{1M} hybrids. Also, the body size of the only F₂ hybrid that survived to adulthood (SVL 129.5mm) was close to the values of the *E. macularius*. The corresponding comparisons of the hatchling body size revealed that the *E. angramainyu* were larger than the *E. macularius* (P = 0.0001) and the hybrids (F₁, F₂, both types of B_{1M}; Ps = 0.0002, 0.0029, 0.0008 and 0.0002, respectively). Moreover, the *E. macularius* hatchlings were slightly, but significantly smaller than both F₁ (P = 0.0373) and a specific category of the B_{1M} hybrids (MAxM, i.e., descendants of F₁ females; P = 0.0115).

Body shape. Canonical variate analysis (CVA) performed on size-adjusted measurements revealed that the body shape differed markedly among the *E. macularius*, *E. angramainyu* and their F₁ hybrids (Fig 6). The first canonical axis discriminating the *E. macularius* from the *E. angramainyu* may be interpreted as a relative length of limbs (the latter species possessing longer limbs; correlations between this axis and limb measurements were: -0.469, -0.353, -0.309, -0.378, and -0.319 for the lengths of femur, tibia, humerus, ulna, and middle finger, respectively), while the second canonical axis discriminating the F₁ hybrids from the parental species correlated with the snout-vent length (r = 0.594) and head width (r = 0.307). The discriminant function analysis (DFA; Wilks' Lambda = 0.178, F_{30, 214} = 9.76, p < 0.0001) revealed that the

Table 3. Means and Standard errors (SE) for Number of spots on the head, Spot size and Spot length in the *E. angramainyu* (P_A), *E. macularius* (P_M), and their F₁ and B_{1M} hybrids. Number of spots on the head—square root transformed, Spot size—area of the largest spot scaled to the head size and natural log-transformed, and Spot length—length of the largest spot scaled to the head length and natural log-transformed. In the case of the Number of spots, post hoc Tukey tests at P < 0.05 were significant for all comparisons. The same procedure revealed two homogenous groups (*E. angramainyu* and F₁; *E. macularius* and B_{1M}) for the Spot area and Spot length. N—number of animals in the testing group.

Group	Number of spots			Spot size			Spot length	
	N	Mean	SE	N	Mean	SE	Mean	SE
P _A	29	2.669	0.203	29	-2.045	0.134	1.192	0.132
F ₁	28	3.618	0.139	28	-2.254	0.117	0.911	0.111
B _{1M}	27	4.731	0.246	27	-3.208	0.167	0.041	0.149
P _M	29	5.767	0.257	27	-3.551	0.123	-0.182	0.104

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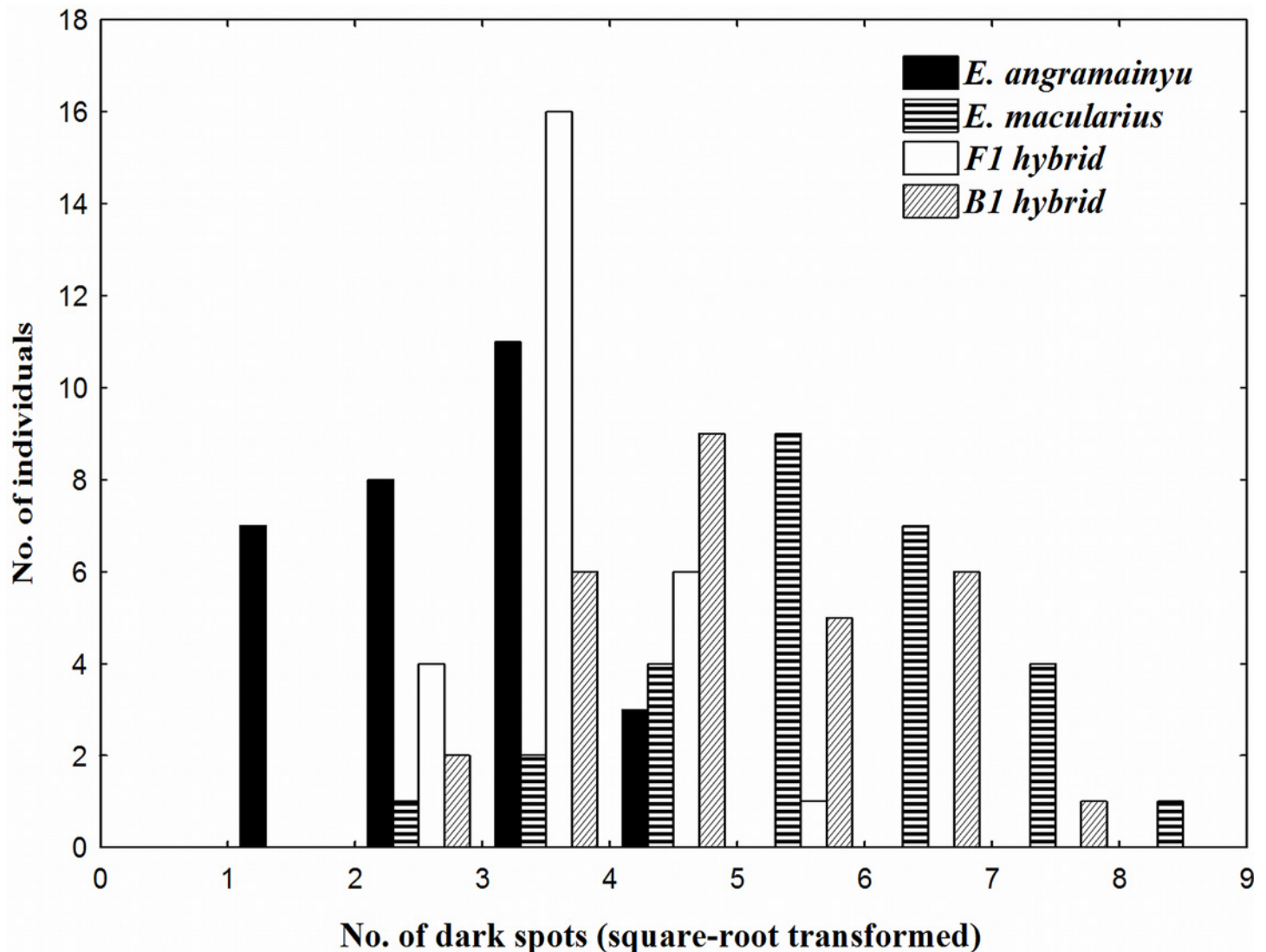


Fig 3. Variation in the number of dark spots on head. *E. angramainyu*, *E. macularius*, and their F₁ and B_{1M} hybrids. The number of spots was square-root transformed.

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overall reclassification success was high (87%), only one of the 29 individuals of the *E. angramainyu* and two of the 68 individuals of the *E. macularius* were assigned to the opposite species according to their body shape. Out of the 27 F₁ hybrids, seven were erroneously assigned to the *E. macularius* and only one to the *E. angramainyu* (see Table 4). Application of the above discriminant functions to the backcrosses and higher order hybrids showed that only one of these animals was classified as an F₁ hybrid; the others were classified either as the *E. macularius* (20 cases) or as the *E. angramainyu* (9 cases).

Discussion

Hybridization success

We demonstrated that the attempts to cross an *E. macularius* with an *E. angramainyu* regularly result in successful copulations, production of fertilized eggs and well-developed hatchlings.

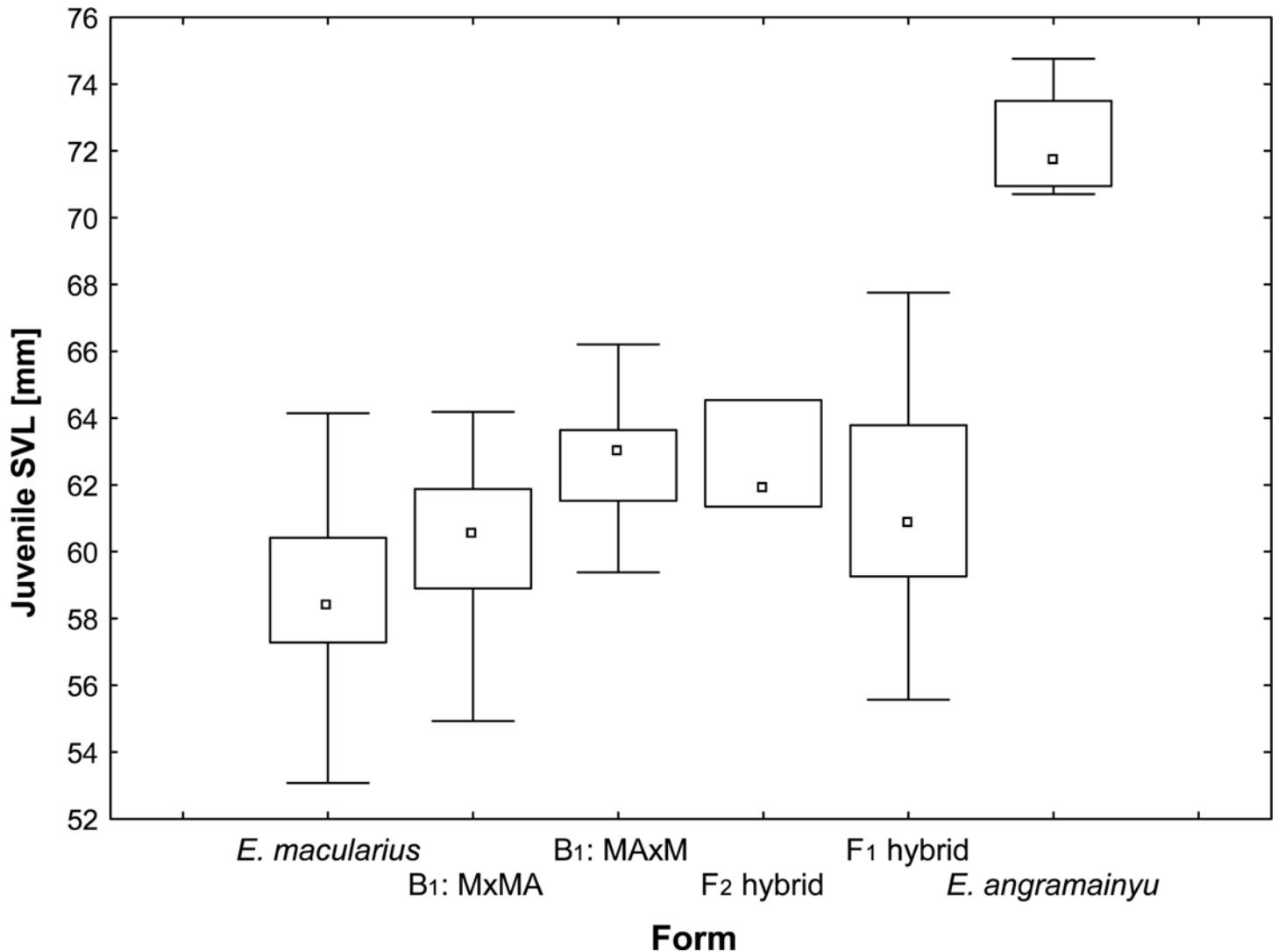


Fig 4. Box plots of hatchling snout-vent lengths. *E. macularius* (n = 32), *E. angramainyu* (n = 4), their hybrids of the first (F₁; n = 25) and second (F₂; n = 3) filial generations and the reciprocal backcrosses of F₁ males or females to the *E. macularius* (B_{1M}; the individuals with father F₁ hybrid are denoted as MxMA, while those with the mother F₁ hybrid as MAxM; n = 11 and 16, respectively). Median, quartiles and ranges are provided.

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Considering that both geological and genetic evidence suggest that the divergence of the *E. macularius* and *E. angramainyu* lasted at least 12–15 million years (see under [Introduction](#)), even the ability to produce healthy F₁ hybrids is remarkable. Comparably, divergent species of mammals are typically unable to produce F₁ hybrids (but see [\[114, 115\]](#)). Thus, our results in the eyelid geckos fit the slow (“avian”) rather than the rapid (“mammalian”) rate of the evolution of postzygotic RIMs [\[44, 47\]](#).

Not the ability to produce F₁ hybrids, but especially the fertility of the hybrids usually determines the evolutionary consequences of hybridization. Bolonick and Near (2005) [\[34\]](#) demonstrated in centrarchid fishes that the divergence time of species still able to produce fertile hybrids was two times shorter than that of those able to produce viable, but sterile F₁ hybrids (15 versus 34 million years, respectively, in a similar way in birds [\[116\]](#)).

In our experiments, most of the F₁ hybrids of the *E. macularius* and *E. angramainyu* appeared fertile when backcrossed with the *E. macularius* (see [Table 1](#)). Also, at least two from

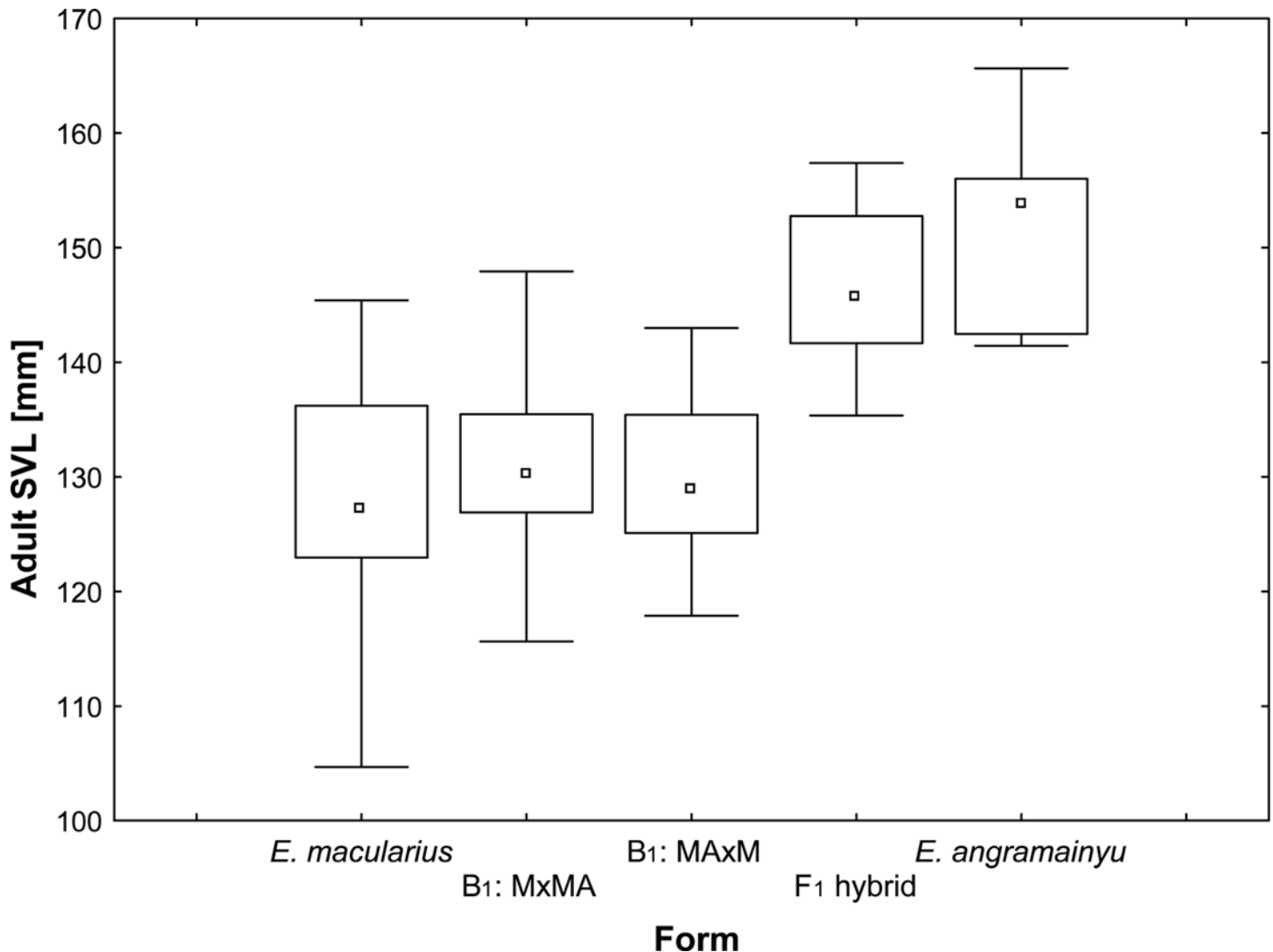


Fig 5. Box plots of adult snout-vent lengths. *E. macularius* (n = 68), *E. angramainyu* (n = 15), their hybrids of the first filial generation (F₁; n = 27), and its reciprocal backcrosses of F₁ males or females to the *E. macularius* (B_{1M}; the individuals with the father F₁ hybrid are denoted as MxMA, while those with the mother F₁ hybrid as MAxM; n = 10 and 17, respectively). Median, quartiles and ranges are provided.

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the five resulting backcrosses were fertile. Thus, low success of attempts to produce F₂ hybrids should be attributed to genetic incompatibility rather than to sterility of the F₁ hybrids. This conclusion also concerns the failed backcrossing to the *E. angramainyu* (see Table 1). In this case, successful copulations initiated laying of eggs, which failed to develop and contained no macroscopically detectable embryos. The likely cause is a defect of either fertilization or early development. The asymmetric pattern of incompatibilities allowing backcrossing of the F₁ hybrids solely to the *E. macularius* is remarkable, but not exceptional. Such asymmetries fit the predictions of some genetic theoreticians [117] and were also previously reported from experiments performed in other animal taxa (e.g., fishes [118], amphibians [119, 120], lizards [69, 121], insects [122]).

Because we have only one breeding male *E. angramainyu*, the failed backcrossing to the *E. angramainyu* could be due to mating between close relatives, F₁ hybrid daughters with the *E. angramainyu* father, respectively. Similarly, the low success of producing F₂ hybrids could be

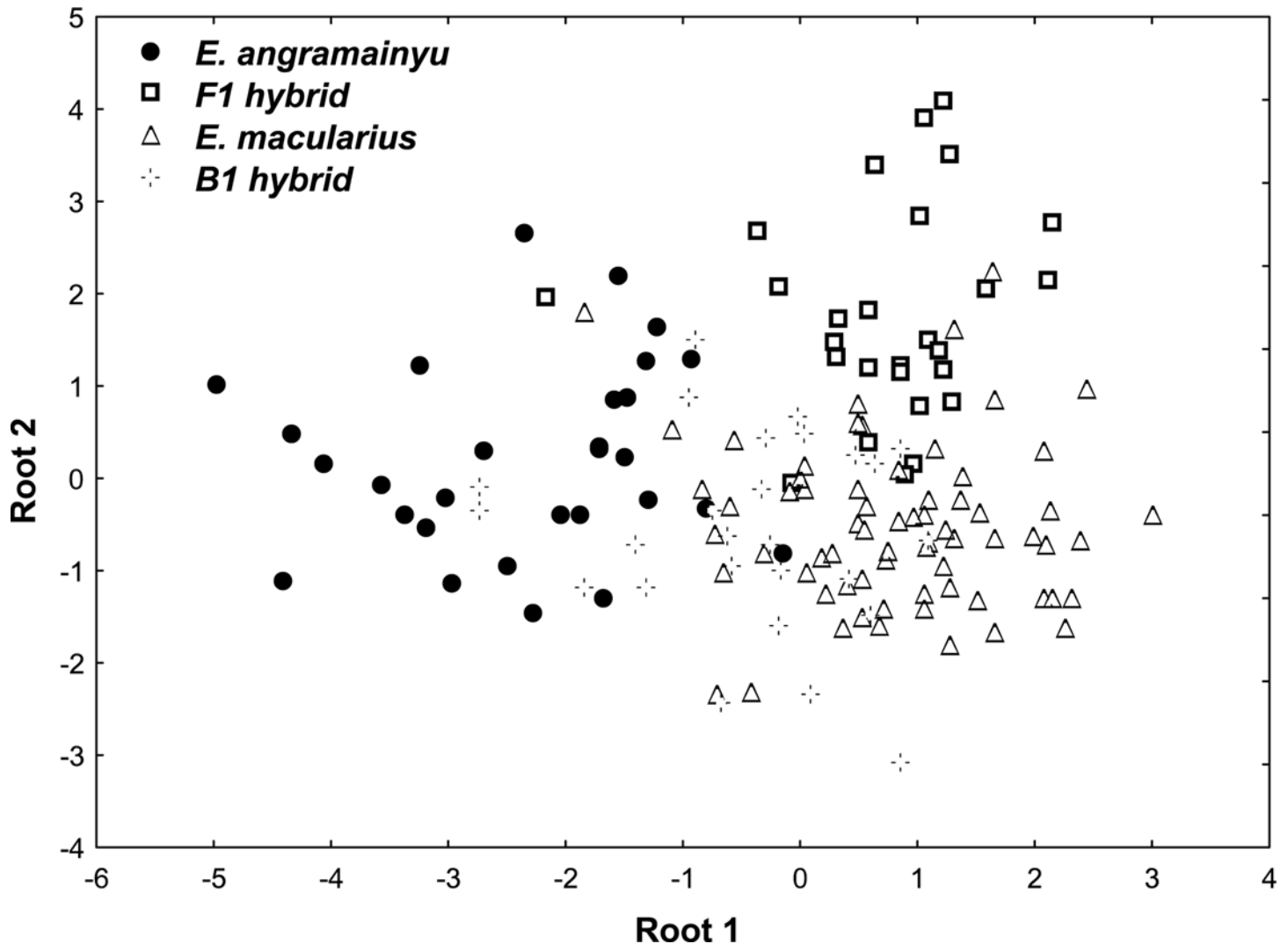


Fig 6. Results of canonical variate analysis extracting multivariate axes (roots 1 and 2). The results discriminated the *E. macularius*, *E. angramainyu*, and their F₁ hybrids from 15 size-adjusted morphometric traits. Backcrosses of the F₁ hybrids with the *E. macularius* were also projected into this morphospace.

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determined by the breeding of siblings. On the other hand, the strong inbreeding impact on the fitness in the first generation of the relative breeding in lizards was supported neither by the studies in the literature [123], nor by our own experience with the breeding of closely related animals of the *E. macularius*. In case of the Swedish sand lizard, Olsson et al. (2002) demonstrated that the sand lizards produce malformed offspring often when they mate with siblings. However, there is low level of genetic variation and there are monitored similarly malformed offspring in this natural population too (up to 10%) [124]. The higher incidence of the malformed offspring through the mating of siblings is probably the result of inbreeding depression of entire population.

The observed difference in hatching success between the parental species (92% in *E. macularius* and 34% in *E. angramainyu*) considerably limits the interpretation of the quantitative differences in hatchability between the parental species and their hybrids. The lower hatching success of the *E. angramainyu* may be attributed to suboptimal incubation conditions. The

Table 4. Results of the discriminant function analysis (DFA) on 15 size-adjusted morphometric traits. The *E. macularius*, *E. angramainyu*, and their F₁ hybrids were included in the analysis. Resulting discriminant functions were then applied to the reclassification of these animals as well as additional ones belonging to other categories of their hybrids into these three groups. The numbers indicate assignment of the individual as predicted by DFA. Generation and Crossing abbreviation = see Table 1. No. of examined individuals = the observed number of animals belonging to each category; Reclassification success = percent of individuals assigned to a correct group.

Generation	Crossing abbreviation	Reclassification success [%]	P _A <i>E. angramainyu</i>	F ₁ hybrid	P _M <i>E. macularius</i>	No. of examined individuals
P _A	A	93	27	1	1	29
F ₁	MA	70	1	19	7	27
P _M	M	91	2	4	62	68
F ₂	MAxMA	-	0	0	1	1
B _{1M}	MxMA	-	3	0	14	17
B _{1M}	MAxM	-	5	1	4	10
B _{2M}	(MxMA)xM	-	1	0	0	1
B _{1M} xP _A	(MxMA)xA	-	0	0	1	1

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optimization of the incubation temperature of this little known species would need huge number of eggs and would require a separate long-term study. Consequently, it is difficult to distinguish between the additive effects of genes and the effects resulting from the incompatibility of genes originating from different parental species that are present in hybrids. On the other hand, it has not shown that the different optimal incubation temperature of the parental species (26°C or 28°C in this study) would affect the hatchability of the eggs produced by the F₁ hybrid females.

Due to an extreme rarity of the *E. angramainyu*, it was impossible to obtain those combinations of reciprocal crosses involving females of this parental species. Nevertheless, in the genus *Eublepharis*, males are genetically fully equivalent to females due to the presence of temperature sex determination (TSD, [125–127]). This genetic equality of the sexes, however, does not mean an exclusion of the maternal effects and/or sex biased effects of DMIs.

In spite of the difficulties to produce F₂ hybrids of the *E. macularius* and *E. angramainyu* and the failed backcrossing of the F₁ hybrids with the *E. angramainyu*, the successful backcrossing of the F₁ hybrids with the *E. macularius* provides a theoretical possibility for introgression of the *E. angramainyu* genes into the populations of the *E. macularius*. This suggests that postzygotic RIMs between these distinct species have not been completed.

Another aspect of successful hybridization is the viability, developmental stability and health of the hybrids. As repeatedly demonstrated in many model taxa [22, 34, 53, 128, 129], the viability of the F₁ hybrids may be comparable or even higher than that of the parental species due to the heterosis and the absence of segregation. In contrast, the negative effects of hybridization on post-hatching viability usually result from segregation, and thus, they are confined to the F₂ generation, backcrosses, and higher order hybrids [21, 130]. In our experiment, the survival rate was high and fairly comparable among the *E. angramainyu*, *E. macularius*, F₁ hybrids and the pooled remaining categories of the hybrids. Nevertheless, all four hatchlings belonging to the F₂ generation showed deformations of the tail suggesting developmental problems during embryogenesis and only one of them survived up to the age of one year. Although the sample size of the F₂ generation was too small to allow for correct comparison of the survival rate, this record is noticeable.

The presence of TSD in the genus *Eublepharis* [125–127], which complicates the evolution of functionally differentiated sex chromosomes [131], may provide an alternative explanation of the geckos' ability to produce fertile between-species hybrids. In many animal taxa with genetic sex determination (GSD), fitness of the hybrids is strongly sex-biased. Following the

empirical Haldane's rule [132], hybrids of a heterogametic sex are regularly more affected by incompatibilities and consequent infertility. The genes responsible for the speciation (DMIs) tend to be recessive and localized on the non-homologous part of the X or Z chromosomes (the sex chromosomes present in a homogametic sex; [31, 133]). Thus, it may be expected that the absence of sex chromosomes retard the evolution of the postzygotic reproductive isolating mechanisms (RIMs). Nevertheless, the list of the reptilian taxa, in which the hybridization among distant species was reported, contains not only clades with the TSD (chelonians, crocodylians), but also many species with the GSD (e.g., iguanids [62–65] and colubrid snakes [75, 134, 135]; for evolution of sex determination mechanisms among squamates see [136]). Surprisingly, a recent review of hybridization events in lizards showed that reliable reports about hybridization of species with TSD are lacking [44]. In this context, the fact that the HKY distance of the mt cyt b gene sequences of the *E. angramainyu* and *E. macularius* (22%) is higher than those in all other pairs of hybridizing lizard species reported to date [44].

Published studies properly documenting experimental hybridization of distinct lizard species are extremely scarce [69, 70, 137–140], for review see [44]. There is, however, a study performed in a model system of European lizards with GSD exhibiting a degree of genetic differentiation [141], which is roughly comparable to the one occurring between the *E. macularius* and *E. angramainyu* possessing TSD. Rykena (1991, 1996, 2002) [67–69] performed experimental crossings among five species belonging to the genus *Lacerta* (*L. viridis*, *L. agilis*, *L. strigata*, *L. schreiberi*, and *L. trilineata*) with well-differentiated sex chromosomes (ZW). The author confirmed a sex bias predicted by the Haldane's rule, i.e., the hybrid infertility affected the heterogametic females, but not the homogametic males of between-species hybrids. The rate of female infertility proved by both breeding and dissection of the reproductive organs varied among pairs of the hybridized species. The attempts to produce F₁ hybrids and backcrosses (via fertile male hybrids) were repeatedly successful, while the F₂ hybrids were only rare. Thus, these thorough experiments demonstrated that a gene flow among the studied species of the genus *Lacerta* is not entirely precluded by postzygotic RIMs in spite of GSD. Consequently, to properly answer the question whether the TSD enhances the success of hybridization between distinct species, additional experimental data are required. Multiple pairs of either TSD or GSD species with similar divergence time need to be crossed and the efficiency of the recorded RIMs compared.

Phenotype of the hybrids

Our morphological analyses confirmed a clear differentiation of the studied populations of the *E. macularius* and *E. angramainyu* in the body size and shape, as well as in the coloration pattern. The phenotype of the descendants of the *E. macularius* mothers sired by *E. angramainyu* (or F₁ hybrid) males contained clear paternal characters. This excludes the theoretical possibility of their parthenogenetic origin instead of hybridization. It is in accord with the absence of any record of parthenogenesis in the family Eublepharidae (for recent records of parthenogenesis in other reptiles, see [142–147]).

As expected, hybrid specimens tend to show intermediate characters, but a resemblance of the hybrid phenotype to the paternal and maternal ones varies among crossings and differs from a trait to a trait. The F₁ hybrids, descendants of an *E. macularius* female and an *E. angramainyu* male, resemble the *E. angramainyu* in their large adult body size, which strongly contrasts with a small body size of the hatchlings (which is close to that of their mothers). This may be interpreted either as dominance of the paternal alleles or as a result of enhanced growth enabled by the heterosis. In contrast, body shape of the F₁ hybrids was close to that of the *E. macularius* along the first canonical axis (CV1; short limbs), but showed a specific feature

(longer SVL and wider head) that differed from both the paternal species as well as the higher order hybrids on the CV2 axis (see Fig 6).

Similar unique characters of hybrids were demonstrated in other taxa like transgression segregation [18, 148–150]. These novelties may be preferred in some ecological conditions (e. g., suboptimal for parent species [22]). In some cases, the hybrids were reported to be possibly more competitive than the parent species (e.g., parthenogenetic species [151], but see [6, 22]). It is known that certain body constitution is optimal for a specific habitat (grassland, rocky land, sand dunes) and is also positively selected for different mobility. Long legs are better for sprint and jumping, short robust legs are favored for burrowing and rock climbing [152–154]. Due to the origin of the transgression characters or intermediate characters of hybrids, these specimens could occupy new ecological niches [155], gain new food sources [22], be better in some performance activities [148, 156], and then be more successful against predators or in male fights over territories and mating rights than one or both of the parental species. Nevertheless, relatively instantaneous combination of traits developed due to hybridization facilitates a rapid adaptive radiation [4, 157] and offers fresh evolutionary scenarios for re-examination in nature selection.

Conclusions

We demonstrated that the *E. macularius* is able to hybridize with its congeneric species, the *E. angramainyu*. F₁ hybrids are viable and fertile, and introgression of the *E. angramainyu* genes into the *E. macularius* genome is enabled via backcrossing. The examined hybrids (except those of the F₂ generation) displayed neither malformations nor reduced survival. Analyses of morphometric and coloration traits confirmed phenotypic distinctness of both parental species and their F₁ hybrids.

These findings contrast with the scenario of a long-term geographic and evolutionary separation of these species, which is supported by both biogeographic and genetic arguments.

In conclusion, occurrence of fertile hybrids of distinct species, which are comparably divergent such as the *E. angramainyu* and *E. macularius*, may be also expected in other taxa of squamates. This would violate the current estimates of species diversity in lizards as well as warn against taxonomic decisions leading to excessive splitting of lizard species.

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Author Contributions

Conceived and designed the experiments: DF EL JLL. Performed the experiments: JLL DF. Analyzed the data: DF JLL. Contributed reagents/materials/analysis tools: DF EL. Wrote the paper: JLL DF EL.

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IV.

Fast or slow? A comparative study of body weight trajectories in three species of the genus *Eublepharis* and their hybrids

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Fast or slow? A comparative study of body weight trajectories in
three species of the genus *Eublepharis* and their hybrids

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Abstract

Despite the extensive research effort devoted to the evolution of life-histories, ontogenetic trajectories and processes underlying the variation in adult body weight remain understudied in some animal taxa. Here we report rates and timing of growth recorded in a common garden experiment. We studied two wild-derived populations of a model lizard species (*Eublepharis macularius*), other two closely related species (*E. angramainyu* and *E. sp.*) and the between-species hybrids.

We monitored growth from hatching to adulthood in 267 geckos. We examined the trajectories of body weight and estimated parameters of logistic growth curve (a , K , T) for each gecko. We detected clear differences among examined species/populations, which can be interpreted in terms of “fast – slow” continuum of life-history strategies. The mean asymptotic body size (a) was the highest in *E. angramainyu* and further decreased in following order: yellow population of *E. macularius*, white population of *E. macularius* and dark *E. sp.* In contrast, the growth rate (K) showed the inverse pattern. Contra intuitively, the largest species exhibited the slowest growth rates. Thus, the final body size was determined namely by inflection point parameter (T). This parameter reflecting duration of the exponential growth period increased with mean asymptotic body size and easily overcompensated the effect of decreasing growth rates in larger species.

Compared to parental species, the F_1 and backcross hybrids of *E. macularius* x *E. angramainyu* and F_1 and F_2 hybrids of *E. macularius* x *E. sp.* exhibited intermediate values of growth parameters. Thus, except the case of F_2 hybrid of *E. macularius* x *E. angramainyu*, we failed to detect deleterious effects of hybridization on growth performance in these animals with temperature sex determination.

Key words: body size – growth rate – fast-slow continuum – hybridization – growth model

Introduction

Body size is a crucial parameter determining ecological and evolutionary attributes of animals (Peters, 1983; Schmidt-Nielsen, 1984). Its phenotypic variation may be caused by both genetic and environmental components (West-Eberhard, 2003). As a result, body size contributes to fitness and varies substantially among individuals, populations and species (Darwin, 1871; Boback, 2003; Fairbairn, 1997). Nevertheless, body size is not just a static trait. A final body size of the animal is a product of ontogenetic trajectory typically involving growth process.

Growth trajectories are perfectly understood in fast growing avian species (Starck and Ricklefs, 1998). Nevertheless, collection of datasets covering entire period of growth is sometimes extremely laborious and time-consuming. It is especially the case of species with slow ontogenetic trajectories and/or indeterminate growers (but see Guarino et al., 2010; Frynta et al., 2010; Roitberg and Smirina, 2006; Haenel and John-Alder, 2002; Shine and Charnov, 1992; Dunham, 1978, Schoener and Schoener, 1978 in squamate reptiles; Ali et al., 2003; Dutta, 1994; Paloheimo and Dickie, 1965; Parker and Larkin, 1959 in fishes). This leads to simplification of the description of body growth as a function of growth increments (typically used in agri- and aqua-culture).

The growth trajectories typically consist of two major components contributing to the final body size. In a typical case, these are an intrinsic growth rate and a duration of exponential growth period. Nevertheless, the latter one is not estimated as a separate parameter by some widely-used theoretical growth models (e.g. von Bertalanffy, West production model), which are applicable even when the data points do not cover the whole course of ontogeny. The logistic growth model (Winsor, 1932, see below) fits very well empirical data concerning detailed description of growth trajectories and produce required information about the duration of exponential growth period further referred to as an

inflection point. The estimates from this model are: growth rate, inflection point and asymptotic body size. Both former two parameters may contribute to the final body size and are inherently intercorrelated.

The growth trajectories and their components (parameters) belong to life-history variables. Besides growth, life-history variables comprise body size, maturation, longevity, curves of mortality rates, reproductive investments etc. In many animal taxa, these variables are tightly intercorrelated and arranged along a common gradient, typically forming an axis from slow to fast life-histories (Bennett and Owens, 2002; Stearns, 1983). Thus, the composite measure of life-histories from a multivariate data set is used to classify the position across the current concept of “fast-slow” continuum (e.g. Stearns, 1983; Gaillard et al., 1989; Bielby et al., 2007; Jones et al., 2008). Nevertheless, even in absence of complex data, it is possible to estimate approximate position of individual species/populations on this axis according to limited number of reliable life-history variables. Under some circumstances, growth rates and/or timing of the growth may be helpful in this respect.

In our study, we monitored three closely related species of eyelid geckos of the genus *Eublepharis*. During the last century, leopard gecko (*Eublepharis macularius*, Blyth, 1854) became the laboratory animal as well as a captive bred pet. It is routinely used as model species for studies of incubation temperature and its hormonal consequences influencing brain development (Coomber et al., 1997; Crews et al., 1996; Crews et al., 1997; Flores and Crews, 1995), antipredator strategies, etc. (Landova et al., 2013; Landova et al., 2016). Eublepharid geckos (Eublepharidae) vary considerably in body size – the largest species *E. angramainyu* (Anderson and Leviton, 1966) is more than 20 times heavier than the smallest *Coleonyx brevis* (Stejneger, 1893). Thus, the family was repeatedly used as a model for studies dealing with evolution of body

size (Kratochvil and Frynta, 2002), parental investment (Kratochvil and Frynta, 2006a; Kratochvil and Frynta, 2006b), growth (Kratochvil and Frynta, 2003), allometries of cell size, DNA content and metabolism (Starostova et al., 2005; Starostova et al., 2009; Starostova et al., 2013).

Growth rates and trajectories may properly reflect life-history strategy of the animal on condition that these parameters are determined solely by underlying trade-offs and corresponding strategic decisions concerning investment. Growth may be, however, constrained by fitness (performance, health status) of the animal. In this respect, efficiency of the growth can serve in monitoring of the processes which are suspect for deleterious effects.

We adopted this approach of comparison the growth parameters to explore the effect of experimental crossing of species/populations of eublepharid geckos. The real effect of hybridization on fitness is still controversial. Both negative as well as positive outcomes were associated with hybridization in natural and experimental conditions (Montanari et al., 2017; Bartley et al., 2000; Jancuchova-Laskova et al., 2015b, for reviews see Burke and Arnold, 2001; Schilthuizen et al., 2011; Chen, 2013; Jancuchova-Laskova et al., 2015a). Historically, natural hybridization was considered as exceptional and erroneous events (Mayr, 1963), but the current increase of literature concerning the importance of hybridization for both speciation and adaptation implies the opposite. Genomic and epigenetic insights into the molecular bases of heterosis are indicating an important role of natural hybridization in the formation of new species. Experimental studies dealing with hybridization covering the observation of real parameters of fitness (fertility, viability, body growth) are still very scarce due to the extreme time demands of such experiments (de Verdal et al., 2014; Hatfield and Schluter, 1999; Jancuchova-Laskova et al., 2015b; Rykena, 2002).

The aims of our study were 1) to compare growth parameters of leopard geckos to demonstrate contrasting life-history strategies of examined parental species/populations; and 2) to compare growth parameters of parental species with parameters of F₁ and F₂ hybrids and subsequent backcrosses to reveal the putative beneficial (heterosis in F₁ generation of hybrids) and/or deleterious (incompatibilities leading to segregation load in F₂ and backcrosses) effects of hybridization on fitness.

Materials and Methods

Experimental animals and their maintenance

The breeding stocks of the parental species were 51 individuals of yellow population of *E. macularius* (M), 40 individuals of white population of *E. macularius* (W), 6 individuals of large-bodied *E. angramainyu* (A) and 39 individuals of *E. sp.*, which we further refer to as dark population (D). The authors of previous studies examining D geckos of the same stock considered the description of *E. fuscus* (Börner, 1981), and referred to this taxon, which is closely related to *E. macularius* sensu stricto, as *E. cf. fuscus* (Starostova et al., 2005; Starostova et al., 2008; Starostova et al., 2009).

The distribution of *E. macularius* cover large territories of Afghanistan, Pakistan and India (Seufer et al., 2005), nevertheless the detailed distribution of white and yellow form is not available. The B, W and D populations were imported directly from Pakistan, but the localities remained unknown. *E. angramainyu* is native in Mesopotamia and SW Iran (Anderson, 1999). M and A are allopatric, their territories are separated by the Iranian Plateau and Zagros Mountains which are at least several million years old (Seufer et al., 2005). Thus, there was long-lasting geographical isolation between *E. macularius* complex and *E. angramainyu* (cf. great sequence divergences between mitochondrial genes; uncorrected p-distances for 303 bp fragment of cyt b gene exceed 19%; Palupčíková, unpublished data).

All experimental parental species (M, W and D) were the first generation of descendants of wild-caught animals. Experimental A were wild-caught as well, a putative locality of origin is Choqa Zanbil, Khuzestan province, Iran, 32°00'N 48°31'E, for more details about the locality see Frynta et al., 1997).

The adult animals were housed individually in glass terrariums (60 x 30 x 20cm or 30 x 30 x 20cm, according to their body size). The floor of each cage was covered with bark substrate. Feeding and drinking dishes, as well as paper shelters were provided. During the laying season, containers with adequately humid coconut substrate for egg deposition were added. The geckos were fed crickets and mealworms dusted with vitamins and minerals (Nutri Mix) weekly; AD3 and E vitamins were provided once per 14 days. The ambient temperature in the breeding room was about 28°C with permanent presence of basking sites in every terrarium to maintain a temperature gradient. During the season of egg-laying (February to September), we check the egg-deposition containers for three times a week. The eggs were placed to the incubator and the temperature was set to 28±0.5 °C, which is an optimal and preferred temperature in *E. macularius* (Bull et al., 1988; Bragg et al., 2000; Landova et al., 2013). The incubation temperature of *E. angramainyu* was set lower (26±0.5 °C) according to our previous experience with incubation of this species. The hatchlings were housed individually in plastic boxes (20 x 20 x 15cm) and were fed exclusively with crickets dusted with vitamins up to the three months of their age.

To acquire F₁ hybrids, females of the selected parental species/population were allowed to copulate with one breeding male of the second parental species/population. The resulting F₁ hybrids were reared to sexual maturity and further bred to obtain F₂ hybrids and/or backcrosses with either parental species. As the geckos of the studied genus *Eublepharis* can store sperm for several months, each experimental female was allowed to

copulate exclusively with a single male during a given mating season (lasting from January/February to July/August). In contrast, males could copulate with multiple females within a single breeding season. From this hybridization, we got 68 viable F₁ hybrids, 36 F₂ hybrids and 27 backcrosses.

All individuals were weighed regularly by a digital balance to the nearest 0.01 g initially once per week (up to the age of 5 month), subsequently twice per month and later once per month.

We studied the following thirteen categories of the parental species/populations, their hybrids and backcrosses that are further referred to as follows (the abbreviations are given in parentheses; on the first place, there is always an abbreviation for a female, then cross (x) with a male on the second position:

1. M - the parental generation of the yellow population of *E. macularius*, both parents belong to the yellow population of *E. macularius* (M);
2. W - the parental generation of the white population of *E. macularius*, both parents belong to the white population of *E. macularius* (W);
3. A - the parental generation of the *E. angramainyu*, both parents belong to *E. angramainyu* (A);
4. D – the parental generation of dark population of the genus *Eublepharis*, both parents belong to this dark population (D);
5. MxA - the first-generation hybrid (F₁), a mother of the yellow population of *E. macularius* (M) and a father of the *E. angramainyu* (A);
6. MxD – the first-generation hybrid (F₁), a mother of the yellow population of *E. macularius* (M) and father of the dark population of *E. sp.* (D) grouped together

- with a mother of the dark population of *E. sp.* (D) and father of the yellow population of *E. macularius* (M), we did not examine the influence of mother;
7. WxD – the first-generation hybrid (F₁), a mother of the white population of *E. macularius* (W) and father of the dark population of the *E. sp.* (D);
 8. MxW – the first-generation hybrid (F₁), a mother of the yellow population of *E. macularius* (M) and father of the white population of *E. macularius* (W) grouped together with a mother of the white population of *E. macularius* (W) and father of the yellow population of *E. macularius* (M), we did not examine the influence of mother;
 9. MAxMA - the second-generation hybrid (F₂), both parents are F₁ hybrids of the yellow population of *E. macularius* and *E. angramainyu* (MxA);
 10. MDxMD – the second-generation hybrid (F₂), both parents are F₁ hybrids of the yellow population of *E. macularius* and dark population of *E. sp.* (MxD);
 11. WDxWD – the second-generation hybrid (F₂), both parents are F₁ hybrids of the white population of *E. macularius* and dark population of *E. sp.* (WxD);
 12. MAxM – the first-generation backcross with the yellow population of *E. macularius*, a mother is an F₁ hybrid (MA) and a father belongs to the yellow population of *E. macularius* (M);
 13. MxMA – the first-generation backcross with the yellow population of *E. macularius* (reciprocal to 12), a mother belongs to the yellow population of *E. macularius* (M) and a father is an F₁ hybrid (MxA)

Experiments were performed in accordance with Czech law implementing all corresponding European Union regulations, and were approved by the institutional animal care and use committee.

Statistical methods

We applied a three-parameter logistic regression model (Equation 1) to analyse the growth trajectories of the overall sample of a given species, hybrids and backcross ones. We previously found that this model (Winsor, 1932) fitted very well the data covering the body growth of reptiles from hatching to the adulthood (Frynta et al., 2010). We used the Levenberg-Marquardt algorithm (with 1000 maximum number of iterations), which minimized the sum of squares between predicted and observed values of growth.

$$\text{Body weight [grams]} = a/(1 + e^{-K(\text{age} - T)}) \quad \text{Equation 1}$$

The growth parameters a is predicting the asymptotic body size, parameter K is estimating the growth rate and the last parameter T is expressing the age at inflection point (i.e. the place where the growth rate is maximal, the growth curve changes from convex to concave and the individuals start to decrease the growth rate). We set the starting values of these parameters as follows: $a = 30$, $K = 0.005$ and $T = 150$. Growth equations were computed separately for each individual. Because the number of females (290) highly exceeded the number of males (35) and the intersexual differences were much smaller than interspecific, we pooled the data of both sexes in distinct groups for comparison between species, hybrids and backcrosses.

Interspecific differences in growth curve parameters were tested with general linear model (ANOVA, post-hoc Tukey's test for unequal N). The effect of growth rate and inflexion point on asymptotic body weight was tested by multiple regression separately for each group. All calculations were performed using STATISTICA, version 6.0 (Statsoft, 2001).

In addition to the comparison of growth parameters revealed from logistic regression model, we compared body weight increments from real measurements

calculated as absolute body weight increment (aBWI = actual body weight - previous body weight) and relative body weight increment (rBWI = aBWI/actual body weight).

Results

The estimated growth parameters for distinct populations/species, their F₁ and F₂ hybrids and backcrosses are presented in Table 1. The logistic regression model fits well our longitudinal growth data as obvious from the Table 1 and Fig. 1.

Comparisons among parental species/populations

Growth parameter *a* significantly differed (Table 1) among distinct species/populations (ANOVA: $F_{3,132} = 88.337$, $P < 0.001$). Moreover, *E. angramainyu* exhibited significantly lower growth rate *K* (ANOVA: $F_{3,132} = 16.3791$, $P < 0.001$) and bigger inflexion point *T* (ANOVA: $F_{3,132} = 37.057$, $P < 0.001$) than the other species/populations. Nevertheless, growth parameters revealed from logistic regression model are intercorrelated. The asymptotic body weight (*a*) was closely correlated by a parameter *T* ($r = 0.64, 0.75$ and 0.65 for yellow, white and dark species/populations, respectively). No such correlation was found between *a* and *K* parameters. The whole course of body growth of distinct species/populations is depicted in Fig. 2.

The changes in mean absolute and relative body weight increments (computed from real body weighting) during the post-hatching ontogeny are depicted in Fig. 3.

Comparison among parental species (*E. angramainyu* and *E. macularius*), their F₁ and F₂ hybrids and backcrosses

Growth parameters significantly differed among parental species of A and M and their F₁ hybrids (ANOVA: a: $F_{(2,81)} = 57.725$, $P < 0.0001$; K: $F_{(2,81)} = 10.0467$, $P = 0.000127$; T: $F_{(2,81)} = 24.9932$, $P < 0.0001$). F₂ hybridization was not successful (except one individuuum, which had poor body growth with the lowest prediction of parameter a = 36.546 g in comparison with parental population and F₁ hybrids). The course of body growth of parental species and F₁ and F₂ hybrids is depicted in Fig. 4. The asymptotic body weight significantly differed in both backcrosses (MAxM and MxMA) from parental species of *E. angramainyu* and F₁ hybrids (ANOVA: $F_{(4,104)} = 29,771$, $P < 0,0001$). Nevertheless, the estimations of asymptotic body mass were similar for both backcrosses (see Table 1).

Comparison among parental species (yellow and white *E. macularius* and dark *E. sp.*) and their F₁ and F₂ hybrids

The growth parameters significantly differed among parental species of M and D and their F₁ hybrids (ANOVA: a: $F_{(3,116)} = 31.980$, $P < 0.0001$; K: $F_{(3,116)} = 9.8493$, $P = 0.000008$; T: $F_{(3,116)} = 5.4302$, $P = 0.001571$). Parental species differed in all growth parameters (all $p < 0.01$). This difference is in accordance with our prediction of the genetic distinctness of these parental species. F₁ hybrids differed in parameter a ($p = 0.019977$ and 0.005097 in comparison with M and D, respectively). Moreover, F₁ hybrids also differed in parameter K in comparison with D ($p = 0.006029$). The inflexion point T was similar in F₁ and F₂ hybrids in comparison with parental species. The course of body growth of parental species and F₁ and F₂ hybrids is depicted in Fig. 5.

The hybridization of W with D revealed the distinctness of parental species again. Growth parameters of F₁ and F₂ hybrids were intermediate with values among parental species (see Table 1).

Discussion

Comparisons among parental species/populations

Leopard geckos of the genus *Eublepharis* are long-living animals (maximum lifespan > 25 years, personal observation) laying multiple clutches per season. The clutches are of invariant size, each consisting of two eggs which are extraordinarily large compared to maternal body (Kratochvil and Frynta, 2006a; Kratochvil and Kubicka, 2007). This places their life-history strategy close to the “slow” end of a “fast-slow” continuum reported in lizards. Nevertheless, our analyses uncovered strong differences in growth trajectories among examined species which are clearly associated with the asymptotic body weight. This suggests that the examined species/population still significantly differ in their position along fast-slow axis.

A three-parameter logistic regression model fitted our long-term data covering the course of ontogeny from hatching to the adulthood very well. Parental species/populations (A, M, W and D) significantly differed in estimated asymptotic body weights and growth rates (except the growth rate and inflexion point, which are similar for two closely related populations of yellow and white form of *E. macularius*).

We found that the growth parameters estimated by logistic regression model are intercorrelated. Asymptotic body weight is tightly predicted by the parameter T. Given the mutual relationship of growth parameters, we decided to compute separately the growth rate expressed as absolute and relative body weight increments. This approach allowed us

to compare real increments of studied species/populations across the ontogeny (Fig. 3) and revealed similar results as those deduced from the estimates of parameters K . The growth rate was contra intuitively the lowest in large *E. angramainyu* and highest in small *E. sp.* The final body size was determined namely by inflection point parameter (T). This parameter reflecting duration of the exponential growth period increased with mean asymptotic body size and easily overcompensated the effect of decreasing growth rates in larger species.

A general life-history relationship described long time ago as a Rosa Lee phenomenon (Lee, 1912; Lee, 1920) may provide explanation for the decrease of the growth rate parameter (K) with asymptotic body size (a) found in our data set. Lee's studies concerning age and growth determination in fishes demonstrated that individuals in a population with slower growth rates suffer less mortality when young which points to the existence of the trade-off between growth rate and survival. This phenomenon was traditionally examined in fishes (Taylor and Methot, 2013; Czerniejewski et al., 2011; Fossen et al., 1999; Walker et al., 1998), but only exceptionally applied in other vertebrates. If further proved in geckos, large-bodied species may be selected to avoiding mortality risk by reduction of the growth rates. Nevertheless, preliminary inspection of our unpublished data sets suggests that within each species/population the fast-growing individuals of leopard geckos tend to suffer equal or even lower rates of juvenile mortality. But, the mortality pattern under laboratory conditions differ from those under natural conditions substantially. Thus, we have to search for alternative explanations for reduced growth rates in larger animals. Metabolic rates may be slightly constrained, e.g., by a positive allometric relationship between erythrocyte size and body size which was demonstrated in lizards including eublepharid geckos (Starostova et al., 2005; Frydlova et al., 2013).

Clear differences detected among examined species/populations can be interpreted in terms of “fast – slow” continuum of life-history strategies. *E. angramainyu* is large-bodied species with slow growth rate. This species attained the body weight close to asymptotic values at the age of about three years, but the first copulation we recorded two years later. To the contrary, *E. macularius* is smaller, grows slowly and mature earlier (1-2 years). The sexual maturation is not known from nature, but it is reasonable to suppose that it takes more time due to the seasonality. However, the clutch size is invariant in eublepharid geckos and the relationship between body size and clutch size is isometric (Kratochvil and Frynta, 2006a).

Comparison among parental species (*E. angramainyu* and *E. macularius*), their F₁ and F₂ hybrids and backcrosses

Similar analysis of growth parameters is ideal for comparison of parental and descendant individuals in experimental crossing of species/populations and may contribute to our knowledge about the influence of hybridization on fitness, viability and competitiveness of F₁ and F₂ hybrids and backcrosses. The positive as well as negative effect of hybridization is discussed in current literature (Pfennig, 2007; Bosworth and Waldbieser, 2014; Yan and Wang, 2010; Johnson et al., 2014; de Verdal et al., 2014). Heterosis effect accompanying hybridization is traditionally used in agriculture and aquaculture, because of increased vigour (e.g. larger body size, faster growth rate, higher reproductive output, enhanced tolerance to environmental conditions). Nevertheless, similar experiments concerning the effect of hybridization on body growth in squamate reptiles are completely missing. The pioneering study of hybridization among species of the genus *Lacerta* (Rykena, 2002) provided first insight to the problematic of hybridization in reptiles. Rykena illustrated amazing data concerning hatchability, survival, fertility and physical deformities of F₁ and F₂ hybrids and backcrosses (Rykena, 2002). Nevertheless, lacertids

have genetic sex determination (Odierna et al., 2001). Conclusions concerning hybridization of GSD species may be different from species with temperature sex determination (TSD). TSD groups are more abundant within the squamate reptiles and is considered as ancestral in Squamata (Pokorna and Kratochvil, 2009). The information about patterns of hybridization in TSD species are completely missing.

Our study is a continuation of long-term project dedicated to the experimental hybridization of eyelid geckos. *E. macularius* has temperature sex determination (Wagner, 1980). Sex determination was not experimentally tested in *E. angramainyu*, but we expect TSD as in *E. macularius*, because closely related *Hemitheconyx caudicinctus* has TSD as well (Pokorna and Kratochvil, 2009). Moreover, the analysis of karyotype in eyelid geckos revealed the absence of sex chromosomes (Pokorna et al., 2010). It implies that the genome is the same in both sexes. Preliminary results concerning the fitness indicators of F₁ and F₂ hybrids and backcrosses were published by Jančúchová-Lásková and her colleagues (Jancuchova-Laskova et al., 2015b). It was demonstrated, that M is able to hybridize with congeneric A and produce viable and fertile hybrids without apparent malformations. Moreover, the introgression of the *E. angramainyu* genes into the *E. macularius* genome is possible via backcrossing. Nevertheless, the observation of growth parameters is crucial for the imagination of real competitiveness of hybrids and backcrosses with parental species and consequent advantages of hybridization. Hybridization produces novel genotypes that may be able to outperform their parental species and persist in unoccupied niches if necessary. The individual fitness and the extent to which hybrids interact with their parents (e.g. assortative mating or differential habitat use) is essential for the evolutionary consequences of hybridization.

Our results of growth parameters revealed that F₁ hybrids are intermediate form between parental species (Fig. 4). The body growth of only one F₂ hybrid was very poor

with asymptotic body size smaller than the parental M. Poor fitness of this F₂ hybrid is in congruence with whole poor hatchability (6%) and viability (25%) of F₂ hybrids demonstrated previously (Jancuchova-Laskova et al., 2015b). The growth parameters unequivocally corroborated that the putative fitness losses affect more hybrids of F₂ generation, which is in accord with the general Dobzhansky-Muller incompatibilities (Dobzhansky, 1936; Dobzhansky and Dobzhansky, 1937; Muller, 1942; Muller, 1940) and the empirical evidence (Turelli and Orr, 2000). MAxM and MxMA backcrosses had better hatchability and survival rate than F₂ hybrids. The egg hatchability also dramatically differed between MAxA backcross and MAxM and MxMA backcrosses. While the latter one was possible to incubate (for details see Jancuchova-Laskova et al., 2015b), hatchability of opposite backcrosses (MAxA) was zero even the females laid eggs regularly. This fact is pointing again on some genetic incompatibilities.

Concerning the growth parameters, backcross MAxM did not differ in asymptotic body size and growth rate from the MxMA backcross. The only difference was in the timing of deceleration of body growth. Nevertheless, these backcrosses attained larger body size than one of the parental species (M). In this case, the effect of hybridization on body growth was positive. The advantages of hybridization for backcrosses are usually expected in increasing of heterozygosity, avoiding the inbred depression and occupying new habitats more successfully (Arnold, 1997).

Comparison among parental species (yellow and white *E. macularius* and dark *E. sp.*) and their F₁ and F₂ hybrids

Parental species of yellow population of *E. macularius* and dark *E. sp.* differed in all growth parameters (all $p < 0.01$). This difference is in accordance with our prediction of the genetic distinctness of these parental species. Hybridization of these two species reveal

similar effect on F₁ hybrids. Growth parameters a and T were intermediate, but the growth rate was close to the smaller *E. sp.* in F₁ hybrids. The success rate of gaining F₂ hybrids was better than in hybridization of M with A. Growth parameters of F₂ hybrids were close to the F₁ hybrids. The distribution of *E. macularius* and *E. sp.* is most probably allopatric, but, the sequence divergence is not so huge as in M vs. A. The growth parameters of backcrosses (MxMD) were not monitored, nevertheless the egg hatchability was high (92%, Landová et al., in prep.).

The crossing of white population of *E. macularius* with dark *E. sp.* revealed intermediate values of growth parameters in F₁ and F₂ hybrids in comparison with parental species. It was not possible to test exactly the differences among F₁ and F₂ hybrids due to the low number of hybrids, but the mean values of estimated growth parameters were comparable. Substantial differences in results of hybridization of M/W with D is probably caused by the body size of parental species (i.e. W is the most similar in body size with D).

In conclusion, we demonstrated, that the growth parameters revealed by the three-parameter logistic regression model described the pattern of body growth of studied species/populations of leopard geckos well. The pattern of body growth supports the “fast-slow” life-history continuum with species growing slowly but attaining large asymptotic body size and vice versa. Based on estimated growth parameters, it is possible to distinguish among these species/populations. We used this approach to study the effect of hybridization on fitness. We enriched our knowledge concerning the ability to hybridize in distinct species of the genus *Eublepharis*, which was previously observed in long-term geographic and evolutionary separated species of *E. macularius* and *E. angramainyu* by the additional experimental crossing of *E. macularius* with *E. sp.* Current approach tested the competitiveness of F₁ and F₂ hybrids and backcrosses by comparison of their body

growth parameters. Our results revealed that growth parameters are intermediate in both F₁ hybrids. Poor fitness of F₂ hybrid (MAxMA) is corroborating the outbreeding depression usually observed in F₂ and other segregating generations of between-species hybrids. Nevertheless, the introgression of A genes into M genome is enabled via backcrossing. This fact is employing the natural hybridization into the concept of species adaptation and speciation. Similar pattern concerning occurrence of fertile hybrids of distinct species may be also expected in other taxa of Squamata.

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Table 1. The estimated values (mean \pm SE) of the asymptotic body mass a (g), growth rate K and inflexion point T (days) and variance explained by a model (R^2) with number of individuals (N) for the examined species, hybrids and backcrosses of eublepharid geckos.

Generation	Species/population	$a \pm SE$	$K \pm SE$	$T \pm SE$	$R^2 \pm SE$	N
P	A	101.135 \pm 4.782	0.0055 \pm 0.0021	381.17 \pm 22.21	0.989 \pm 0.003	6
P	W	39.461 \pm 1.852	0.0153 \pm 0.0008	167.68 \pm 8.6	0.985 \pm 0.001	40
P	D	30.44 \pm 1.876	0.0194 \pm 0.0008	140.79 \pm 8.71	0.991 \pm 0.001	39
P	M	49.188 \pm 1.64	0.0144 \pm 0.0007	182.96 \pm 7.62	0.987 \pm 0.001	51
F1	WxD	35.851 \pm 4.782	0.0149 \pm 0.0021	145.14 \pm 22.21	0.98 \pm 0.003	6
F1	MxW	43.764 \pm 2.928	0.0151 \pm 0.0013	160.95 \pm 13.6	0.988 \pm 0.002	16
F1	MxA	78.501 \pm 2.254	0.0108 \pm 0.001	264.14 \pm 10.47	0.981 \pm 0.002	27
F1	MxD	40.484 \pm 2.687	0.0137 \pm 0.0012	168.37 \pm 12.48	0.986 \pm 0.002	19
F2	WDxWD	34.243 \pm 4.427	0.019 \pm 0.002	136.59 \pm 20.57	0.991 \pm 0.003	7
F2	MWxMW	38.483 \pm 2.841	0.0187 \pm 0.0013	137.92 \pm 13.2	0.989 \pm 0.002	17
F2	MAxMA	36.546 \pm 11.713	0.0102 \pm 0.0052	178.52 \pm 54.41	0.984 \pm 0.008	1
F2	MDxMD	36.87 \pm 3.532	0.0195 \pm 0.0016	135.19 \pm 16.41	0.992 \pm 0.002	11
B	MAxM	45.199 \pm 3.704	0.0131 \pm 0.0016	155.54 \pm 17.21	0.98 \pm 0.003	10
B	MxMA	57.347 \pm 3.024	0.0115 \pm 0.0013	202.94 \pm 14.05	0.984 \pm 0.002	15
B	MxMD	40.595 \pm 8.282	0.0206 \pm 0.0037	128.34 \pm 38.47	0.991 \pm 0.006	2

Footnote.

Abbreviations: (P) Parental generation, (F1) the first and (F2) the second filial generation hybrids, (B) the first-generation backcross, (M) the parental generation of the yellow population of *E. macularius*, (W) the parental generation of the white population of *E. macularius*, (A) the parental generation of the *E. angramainyu*, (D) the parental generation of dark population of the genus *Eublepharis*, (MxA) the first-generation hybrid, a mother of the yellow population of *E. macularius* and a father of the *E. angramainyu*, (MxD) the reciprocal first-generation hybrid, a mother/father of the yellow population of *E. macularius* and mother/father of the dark population of the *E. sp.* (WxD) – the first-generation hybrid, a mother of the white population of *E. macularius* and father of the dark population of the *E. sp.*, (MxW) – the reciprocal first-generation hybrid, a mother/father of the yellow population of *E. macularius* and mother/father of the white population of *E. macularius*, (MAxMA) - the second-generation hybrid, both parents are F₁ hybrids of the yellow population of *E. macularius* and *E. angramainyu*, (MDxMD) the second-generation hybrid, both parents are F₁ hybrids of the yellow population of *E. macularius* and dark population of the *E. sp.*, (WDxWD) the second-generation hybrid, both parents are F₁ hybrids of the white population of *E. macularius* and dark population of the *E. sp.*, (MAxM) the first-generation backcross with the yellow population of *E. macularius*, a mother is an F₁ hybrid of yellow population of *E. macularius* and *E. angramainyu* and a father belongs to the yellow population of *E. macularius*, (MxMA) the first-generation backcross with the yellow population of *E. macularius* (reciprocal to previous), a mother belongs to the yellow population of *E. macularius* and a father is an F₁ hybrid of yellow population of *E. macularius* and *E. angramainyu*. On the first place, there is always an abbreviation for a female, then cross (x) with a male on the second position.

Figure 1. Histogram of variance explained (R^2) by a logistic regression model for all studied individuals.

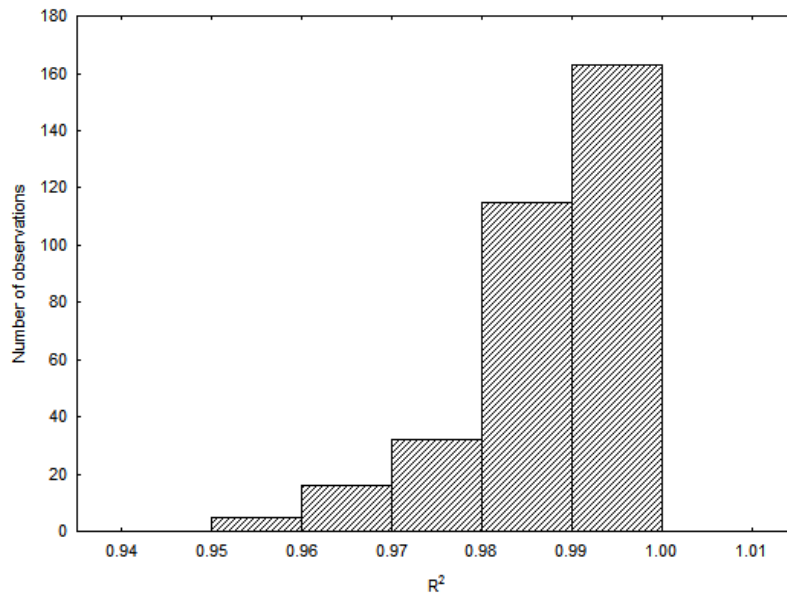


Figure 2. Mean body weight as a function of age predicted by the logistic growth model in studied species of eyelid geckos. Growth parameters were estimated from pooled records of either species/populations. Dotted curves are ± 95 confidence intervals for means of studied species/populations. Abbreviations: (M) yellow population of *E. macularius*, (W) white population of *E. macularius*, (A) *E. angramainyu*, (D) dark population of *E. sp.*

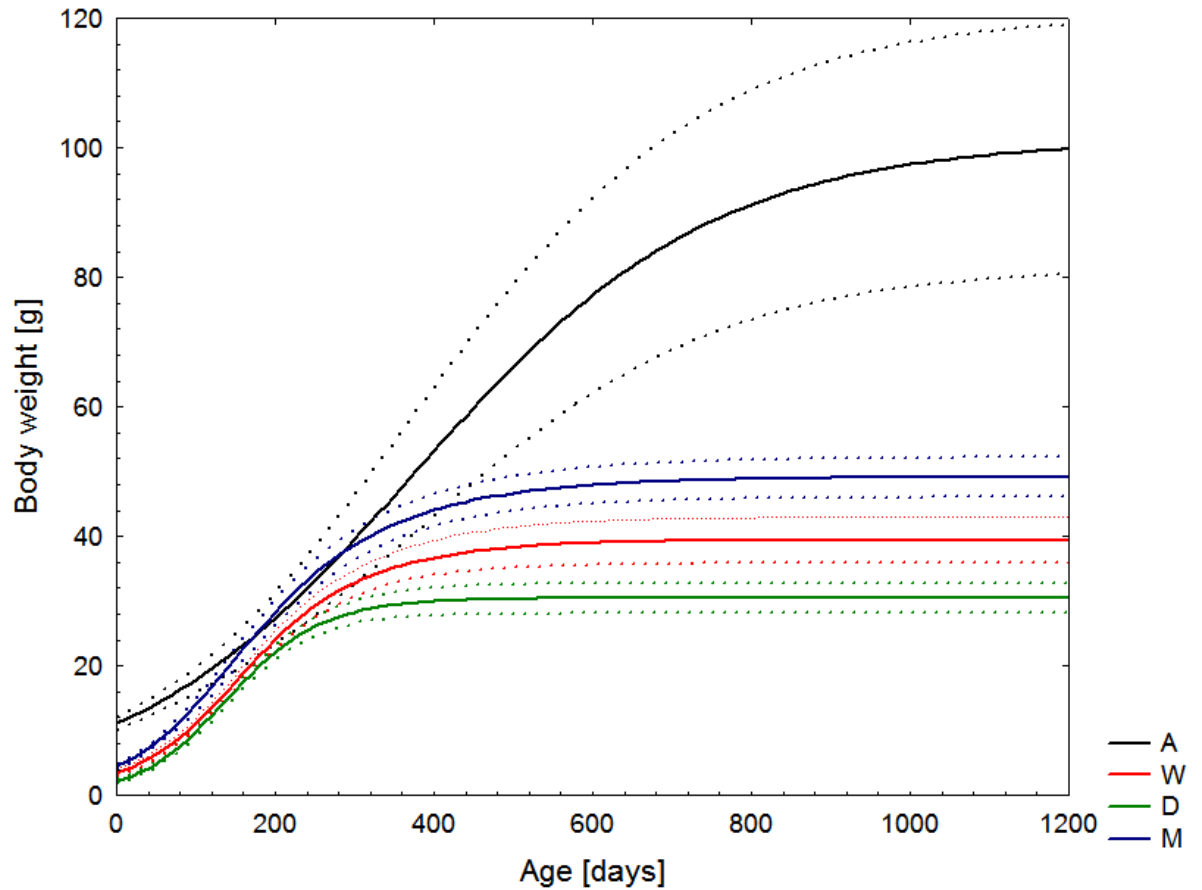


Figure 3. Absolute body weight increments (aBWI) as a function of age (A) and relative body weight increments (rBWI) as a function of actual body weight for distinct species/populations (B). Abbreviations: (M) yellow population of *E. macularius*, (W) white population of *E. macularius*, (A) *E. angramainyu*, (D) dark population of *E. sp.*

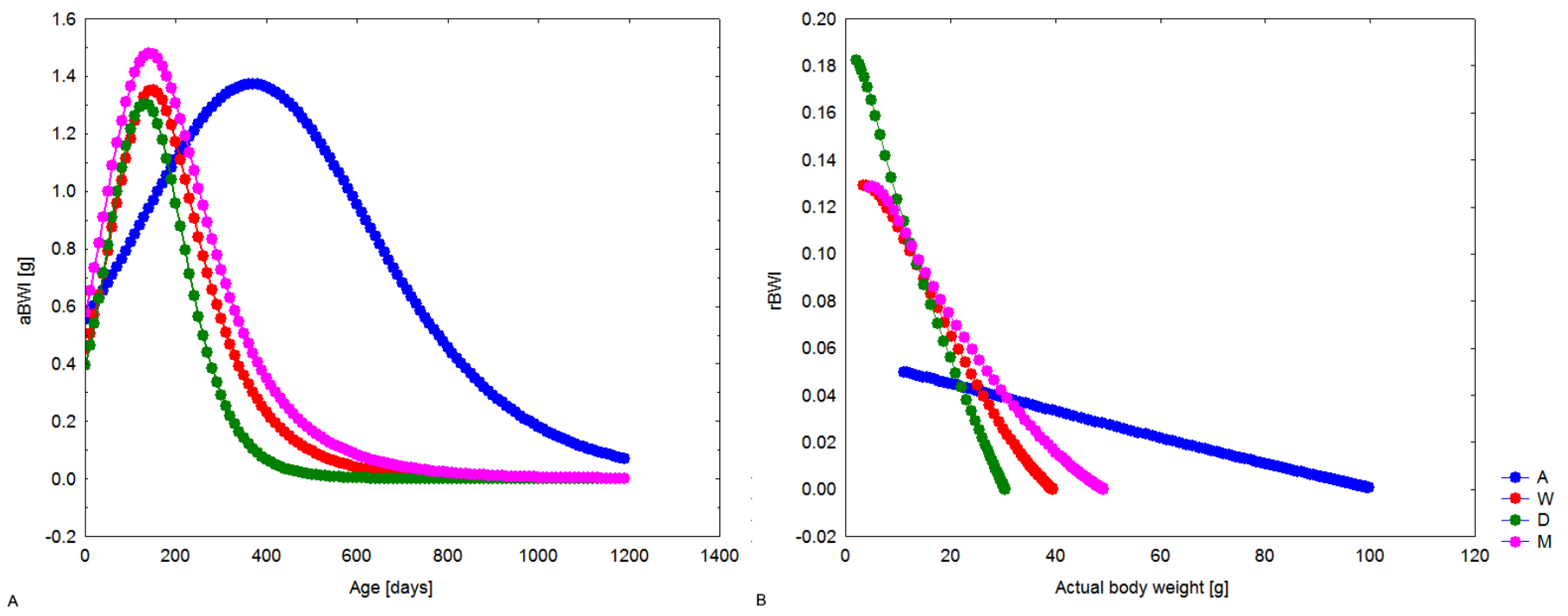


Figure 4. Mean body weight as a function of age predicted by the logistic growth model in parental species of yellow population of *E. macularius* (M) and *E. angramainyu* (A) and their F₁ (MxA) and F₂ (MAxMA) hybrids. Growth parameters were estimated from pooled records of either species and hybrids. Dotted curves are ± 95 confidence intervals for means of studied groups. Note the growth curve of F₁ hybrids (N = 27), which is between the curves of parental species and the poor growth of F₂ hybrid.

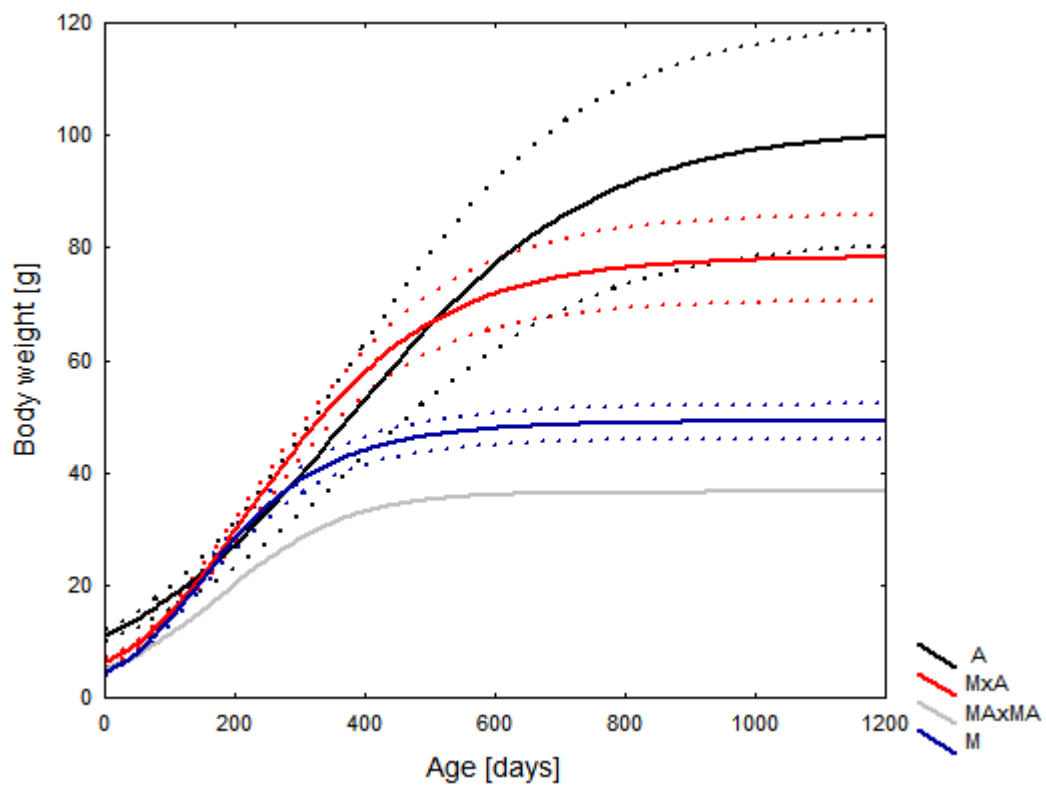
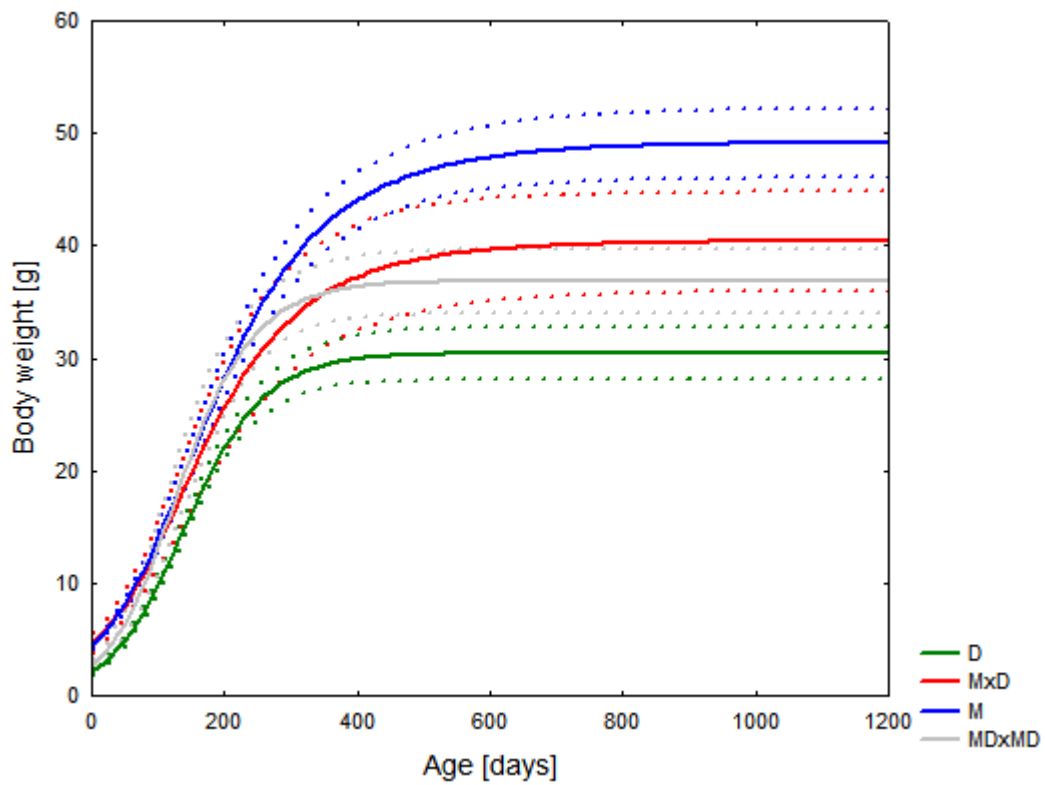


Figure 5. Mean body weight as a function of age predicted by the logistic growth model for parental species of yellow population of *E. macularius* (M), dark population of *E. sp.* (D), F₁ (MxD) and F₂ (MDxMD) hybrids. Growth parameters were estimated from pooled records of either species and hybrids. Dotted curves are ± 95 confidence intervals for means of studied groups. Note the growth curves of F₁ (N = 19) and F₂ (N = 11) hybrids, which are between the curves of parental species.



V.

**Ontogenetic switch between alternative antipredatory strategies in
the leopard gecko (*Eublepharis macularius*): defensive threat
versus escape**

Eva Landová, Jitka Jančúchová-Lásková, Veronika Musilová, Štěpánka
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Příloha

Následující publikace není přímo součástí předkládané disertační práce, protože bude použita k obhajobě disertační práce spoluautorky Petry Suchomelové. Je zde ale uvedena, aby doplnila obraz publikační činnosti autorky.

Experimental assessment of social interactions in two species of the genus *Teratoscincus* (Gekkota)

Petra Suchomelová, Jitka Jančúchová-Lásková, Eva Landová, Daniel Frynta

Behavioural Processes (2015) 120:14-24