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Reinforcement and the Proliferation of Species

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Abstract

Adaptive radiations are characterized by the rapid proliferation of species. Explaining how adaptive radiations occur therefore depends, in part, on identifying how populations become reproductively isolated—and ultimately become different species. Such reproductive isolation could arise when populations adapting to novel niches experience selection to avoid interbreeding and, consequently, evolve mating traits that minimize such hybridization via the process of reinforcement. Here, we highlight that a downstream consequence of reinforcement is divergence of conspecific populations, and this further divergence can instigate species proliferation. Moreover, we evaluate when reinforcement will—and will not—promote species proliferation. Finally, we discuss empirical approaches to test what role, if any, reinforcement plays in species proliferation and, consequently, in adaptive radiation. To date, reinforcement's downstream effects on species proliferation remain largely unknown and speculative. Because the ecological and evolutionary contexts in which adaptive radiations occur are conducive to reinforcement and its downstream consequences, adaptive radiations provide an ideal framework in which to evaluate reinforcement's role in diversification.

Understanding how new species arise is fundamental to explaining adaptive radiations. Yet, understanding speciation per se is potentially insufficient for explaining adaptive radiations. Adaptive radiations are characterized by *species proliferation*—the rapid accumulation of species within a taxonomic group (Simpson 1953; Guyer and Slowinski 1993; Schluter 2000; Gavrilets and Losos 2009; Glor 2010). Thus, the challenge is to explain not only why speciation occurs, but why the process is prolific in some circumstances or lineages but not others.

A variety of ecological and evolutionary factors combine to generate the remarkable phenotypic and ecological diversity that characterize adaptive radiations (Simpson 1953; Schluter 2000; Seehausen 2004; Losos and Mahler 2010; Yoder et al. 2010; Wagner et al. 2012; Bouchenak-Khelladi et al. 2015; Pease et al. 2016; Stroud and Losos 2016; Richards and Martin 2017; Gillespie et al. 2020). In conjunction with these factors, the evolution of traits related to mating (hereafter "mating traits") could play a key role in

adaptive radiations (Seehausen and van Alphen 1999; Wagner et al. 2012). Because lineages experiencing adaptive radiation might be adapting to new niches in the absence of physical barriers to gene flow (Gavrilets and Losos 2009; Rundell and Price 2009; Glor 2010; Losos and Mahler 2010; Simoes et al. 2016), mating traits could generate reproductive isolation (and, ultimately, speciation) between them (Seehausen and Van Alphen 1999). Indeed, mating traits sometimes serve as the sole or most important reproductive isolating mechanisms between species (Coyne and Orr 2004; Ritchie 2007; Price 2008), and the evolution of mating traits that reproductively isolate populations during the early stages of an adaptive radiation could help explain how species proliferate (Stelkens et al. 2010). In the absence of mating traits that isolate such populations, diversification could break down if interbreeding homogenizes populations (Seehausen et al. 1997; Behm et al. 2010). Thus, knowing how mating traits diversify and generate reproductive isolation could be an important component to understanding adaptive radiations.

If incipient species have already diverged (e.g., because they have adapted to different niches), then hybridization between them is potentially deleterious. Consequently, natural selection would tend to favor the evolution of mating traits that minimize the likelihood of hybridization. This process by which traits evolve as an adaptive response to selection minimizing deleterious hybridization between species or incipient species is termed reinforcement (see Table 1 for definitions of key terms used throughout). Historically, reinforcement's role in speciation has been controversial (reviewed in Servedio and Noor 2003; Coyne and Orr 2004; Pfennig and Pfennig 2012). Nevertheless, theory and empirical work support a role for reinforcement in the speciation process because it acts to enhance reproductive isolation between groups that have already begun to diverge (reviewed in Servedio and Noor 2003; Coyne and Orr 2004; Pfennig and Pfennig 2012).

Adaptive radiations provide ecological and evolutionary conditions that are particularly conducive for reinforcement. Specifically, radiating lineages are newly diverged from a common ancestor, so they potentially share similar mating traits that put them at risk of hybridization (Basolo 1995; Coyne and Orr 2004; Gholamhosseini et al. 2013; Willis et al. 2014). Moreover, these lineages might not be separated by distance or other dispersal barriers if they are adapting to fill novel niches in sympatry (Schliewen et al. 1994; Gavrilets and Losos 2009). Consequently, mating interactions and interbreeding between such lineages could be common unless mating traits prevent these interactions. Perhaps most critically, any such interbreeding might be deleterious if it breaks down locally adapted or niche-specific genetic combinations (Hatfield and Schluter 1999; Nosil et al. 2003; Rundle and Nosil 2005; Nosil 2012). In such circumstances, natural selection favoring mating traits that reduce the risk of hybridization could be strong. Indeed, when a risk of hybridization exists, selection against interbreeding could be positively associated with niche novelty or specialization: as niche use becomes more novel or specialized relative to other populations, selection against interbreeding between populations should become stronger (Pfennig and Pfennig 2010; Nosil 2012).

Reinforcement generates 2 hallmark patterns of mating trait diversity. First, mating traits between the hybridizing species diverge within sympatric populations (e.g., Littlejohn 1965; Snell and Hawkinson 1983; Levin 1985; Saetre et al. 1997; Höbel and Gerhardt 2003; Nosil et al. 2003; Higgie and Blows 2007; Kozak et al. 2015). Second, mating traits *within* a given species can also diverge in sympatric populations versus allopatric populations (Figure 1; e.g., Höbel and Gerhardt 2003; Hoskin et al. 2005; Lemmon 2009; Hopkins and Rausher 2014; Pfennig and Rice 2014). This latter pattern arises because selection to avoid hybridization with heterospecifics only occurs in sympatric populations where the 2 species actually co-occur (see Table 1 for definition of heterospecifics). Critically, the divergence in mating traits within a species between sympatric and allopatric populations sets the stage for future speciation events (Figure 1). In particular, if divergent mating traits generate reproductive isolation between conspecific populations in sympatry and allopatry, then a *downstream consequence* of reinforcement is the initiation of speciation between sympatric and allopatric populations (Figure 1). However, whether reinforcement and its downstream effects, if any, promote species proliferation remains an open, and potentially controversial, issue (cf., Abbott et al. 2013; Barton 2013; Servedio et al. 2013).

In this article, we discuss when reinforcement is—and is not—likely to contribute to species proliferation during adaptive radiations. We then describe approaches to assess reinforcement's role in species proliferation. Our goal is to motivate empirical and theoretical work that critically evaluates what role, if any, reinforcement plays in species proliferation generally and adaptive radiation specifically.

When Will Reinforcement Lead to Species Proliferation—and When Will It Not

Extensive data show that reinforcement occurs, resulting in divergent mating traits both between incipient species in sympatric populations and within species between populations in sympatry versus allopatry (reviewed in Servedio and Noor 2003; Coyne and Orr 2004; Pfennig and Pfennig 2012). The issue that arises is whether these patterns of divergence in mating traits actually produce reproductive isolation that contributes to speciation.

In terms of reinforcement between incipient species (Table 1), although the conditions under which reinforcement occurs remain an open question (Marie Curie Speciation Network 2012), theory and empirical work reveal that reinforcement can indeed enhance reproductive isolation between incipient species and thereby contribute to

Table 1. Glossary of terms used in the text

Term	Definition used in text
Species proliferation	The rapid accumulation of species within a taxonomic group.
Incipient species	Groups of organisms that are partly reproductively isolated from one another, but may not be completely reproductively isolated (and are therefore not yet "true" species according to the biological species concept). In Figure 1, white and black birds belong to different incipient species.
Reinforcement	The process by which traits evolve as an adaptive response to selection minimizing deleterious hybridization between species or incipient species
Conspecific	Individual of the focal species or incipient species
Heterospecific	Individual of a different species or incipient species from the focal species or incipient species
Hybridization	Interbreeding between species or incipient species
Sympatry[a]	An area of co-occurrence of different species or incipient species undergoing reinforcement
Allopatry[a]	An area where one species or incipient species occurs without the other(s)
Conspecific populations	Populations belonging to the same focal species or incipient species. As a downstream consequence of reinforcement, conspecific populations might (or might not) become reproductively isolated from one another. In Figure 1, birds of the same color have conspecific populations in sympatry and allopatry.

[a]Sympatry and allopatry are both used as relative terms in this review, because a focal species or incipient species might co-occur with different communities of heterospecifics in different populations. We have tried to be explicit in describing such scenarios.
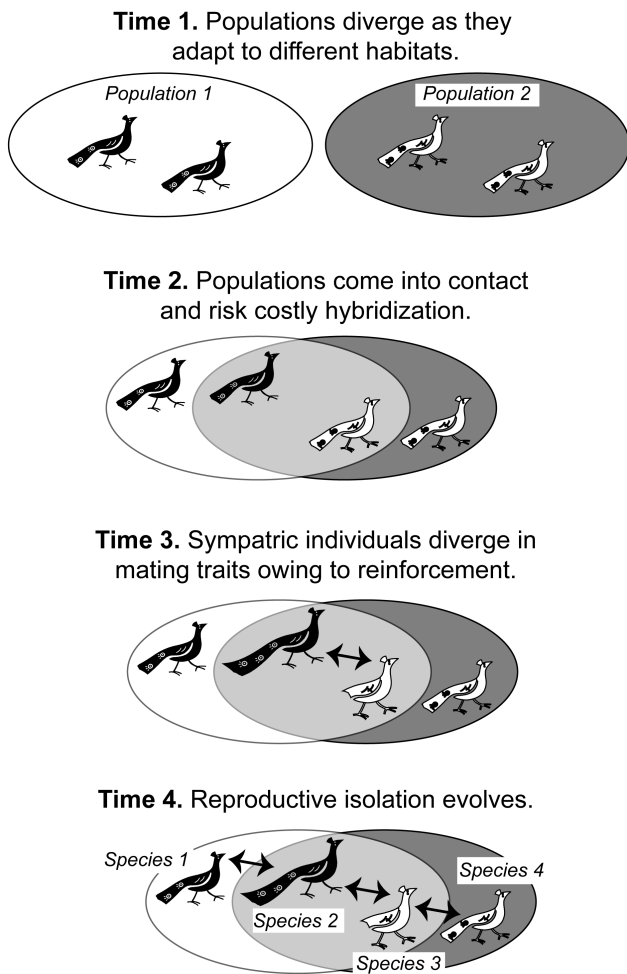
Time 1. Populations diverge as they adapt to different habitats.

Time 2. Populations come into contact and risk costly hybridization.

Time 3. Sympatric individuals diverge in mating traits owing to reinforcement.

Time 4. Reproductive isolation evolves.

Figure 1. Reinforcement's role in species proliferation. When separate populations adapt to local environment or niches (time 1), they can risk costly hybridization if they share similar mating traits—tail length in this example (time 2). Selection will therefore favor the evolution of traits that minimize interbreeding, a process termed reinforcement (time 3; reinforcement is indicated by divergence in tail length). As a consequence of reinforcement, populations 1 and 2 become increasingly reproductively isolated, thereby finalizing speciation between them. Critically, in both lineages, sympatric and allopatric populations become divergent in mating traits. If this divergence reduces mating between the populations, reproductive isolation can result and new species arise. Thus, species proliferate such that, in this example, 4 species arise from 2 initially diverged populations. Table 1 provides both definitions of all terms used here and throughout text; it also describes how use of terms corresponds to the example depicted in this figure. Figure modified and used with permission from Pfennig and Pfennig (2012).

speciation (Servedio and Noor 2003; Pfennig and Pfennig 2012). By contrast, whether divergent mating traits between conspecific populations (Table 1) in sympatry versus those in allopatry initiate sufficient reproductive isolation to cause speciation between them needs to be fully tested (reviewed in Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009; Hoskin and Higgie 2010; Pfennig and Pfennig 2012; Abbott et al. 2013; Pfennig 2016).

Support for the hypothesis that reinforcement can initiate speciation between sympatric versus allopatric populations often takes the form of mate preference tests showing that sympatric individuals preferentially mate with members of their own population as opposed to allopatric individuals (Nosil et al. 2003; Hoskin et al. 2005; Jaenike et al. 2006; Porretta and Urbanelli 2012; Kozak et al. 2015).

Such data are often also combined with genetic analyses that reveal both premating isolation between sympatric and allopatric populations and genetic differentiation of the 2 populations types. For example, Hoskin et al. (2005) evaluated reinforcement and its downstream effects in the green-eyed treefrog (*Litoria genimaculata*). They found that, as a downstream consequence of reinforcement: male mating calls had diverged between populations in sympatry versus allopatry; females preferred males of their own population type; and the populations were genetically distinct. They therefore concluded that reinforcement could indirectly cause speciation between sympatric and allopatric populations (Hoskin et al. 2005).

Such data provide compelling evidence for the possibility that reinforcement between incipient species in sympatric populations can concomitantly generate downstream divergence between sympatric and allopatric populations as depicted in Figure 1 (see also Table 1). Other systems show similar patterns (reviewed in Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009, 2012; Pfennig 2016). Yet, a key issue with the hypothesis that reinforcement between incipient species in sympatric populations will also generate reproductive isolation between conspecific populations in sympatry versus allopatry is that divergent mating traits do not necessarily result in reproductive isolation. In other words, divergent mating traits do not reduce gene flow axiomatically (Pfennig 2016). For example, selection against hybridization can drive sympatric individuals to accept only those mates whose sexual signals do not overlap with heterospecifics' signals. If the range of signals accepted in sympatry is simply a subset of the signals preferred in allopatry (Hudson and Price 2014), then sympatric individuals might be unlikely to mate with those from allopatry, but allopatric individuals would continue to accept mates from sympatric populations (Jaenike et al. 2006; Lemmon 2009; Kozak et al. 2015). Depending on migration patterns, reproductive isolation is unlikely to arise in such a case. Moreover, even if mating traits diverge and do reduce gene flow, the amount of reduction might be insufficient to instigate speciation (sensu Hendry 2009).

Under what conditions might conspecific populations in sympatry become reproductively isolated from conspecific populations in allopatry as a downstream consequence of reinforcement occurring between species in sympatry? Generally, mating traits generate reproductive isolation among populations when migrants into a new population fail to reproduce as successfully as residents; that is, migrants have mating traits (whether mate preferences or sexual signals) that are selectively disfavored relative to residents (Nosil et al. 2003). Such a scenario is most likely when migrants and residents derive from populations with opposing patterns of selection on mating traits (Boughman 2001; Nosil et al. 2003). When reinforcement occurs, allopatric traits that increase the risk of hybridization would be disfavored in sympatry. For reinforcement to result in opposing selection on mating traits between populations in sympatry and allopatry, mating traits that evolve via reinforcement in sympatry must be disfavored in allopatry (Pfennig 2000; Hopkins et al. 2014). Such a pattern is especially likely to arise when sexual signals that are indicative of mate quality in allopatric populations resemble sexual signals possessed by heterospecifics in sympatric populations (recall definition of heterospecifics in Table 1; Pfennig 1998; e.g., Pfennig 2000; Rosenthal et al. 2002; Hankison and Morris 2003; Gumm and Gabor 2005). This scenario generates opposing mating preferences in sympatric versus allopatric populations because mate preferences in sympatric populations are driven one direction by reinforcement, whereas mate preferences in allopatric populations are driven in the opposite direction by selection

to choose high-quality mates (Pfennig 2000; Pfennig and Rice 2014; Calabrese et al., in preparation).

Adaptive radiations could generate the likely conditions for such mate quality–species identity tradeoffs that select for opposing mating traits between sympatric and allopatric populations. Specifically, sympatric *and* allopatric conspecific populations are potentially undergoing adaptive evolution during adaptive radiations. Because sexual selection often favors mating traits that enhance or complement ecological adaptations (Boughman 2002; Podos and Nowicki 2004; Huber et al. 2007; Van Doorn et al. 2009), sexual selection on mating traits in allopatry could be poised to counter reinforcing selection on mating traits in sympatric populations if populations in sympatry and allopatry are adapting to different ecological conditions. Thus, divergent and opposing ecological and sexual selection might make reinforcement especially important in species proliferation during adaptive radiations. However, whether and how mating traits evolve in response to these selective pressures will depend on a variety of ecological, evolutionary, and behavioral factors discussed below.

Ecological Factors Impacting the Likelihood that Reinforcement Leads to Species Proliferation

Five types of ecological factors impact whether or not species proliferation results from reinforcement's effects on mating traits: 1) environmental features that impact gene flow and population isolation; 2) distributions of heterospecifics; 3) community composition; 4) environmental factors that impact the costs of hybridization in sympatry; and 5) the nature of sexual selection in allopatry. We address each of these in turn.

Any environmental features that reduce gene flow and increase isolation of sympatric and allopatric populations will enhance the likelihood of species proliferation as a downstream consequence of reinforcement (Abbott et al. 2013; Comeault and Matute 2016). Where distance between sympatric and allopatric populations is high relative to dispersal distance, or where habitat between sympatric and allopatric populations is unsuitable for migrants, species proliferation as a downstream effect of reinforcement acting in sympatric populations should be more likely. Indeed, examples of reinforcement-initiated reproductive isolation between sympatric and allopatric populations primarily come from systems in which dispersal between the population types (or between different sympatric populations) is restricted (Hoskin et al. 2005; Porretta and Urbanelli 2012; Pfennig and Rice 2014). If, however, gene flow is already low between sympatric and allopatric populations owing to low dispersal, then isolation by distance or vicariance—as opposed to reinforcement in sympatry per se—might be the cause of reproductive isolation between conspecific populations in sympatry and allopatry (Barton 2013; Servedio et al. 2013). Physical isolation versus reinforcement-generated divergent mating traits are not mutually exclusive explanations for reduced gene flow in sympatry versus allopatry. Nevertheless, such alternatives to reinforcement should be considered in ascribing reduced gene flow to reinforcement's effects (Pfennig and Rice 2014; reviewed in Pfennig 2016; see also Lemmon and Juenger 2017).

A second ecological factor that impacts the likelihood that reinforcement will lead to species proliferation is the distribution of heterospecific species (McPeek and Gavrilets 2006; Abbott et al. 2013; recall Table 1 for definition of heterospecifics). If heterospecifics are distributed patchily, reinforcement could drive divergence between sympatric and allopatric populations at each

boundary (Hoskin et al. 2005; Lemmon 2009). Because reduced mating between allopatric and sympatric populations reduces gene flow among patches, each of these diverged populations could form new species (Hoskin and Higgie 2010). Moreover, when heterospecifics are distributed patchily, different sympatric populations may evolve different mating traits in response to reinforcement (Hoskin et al. 2005; Lemmon 2009). In this case, mating trait divergence between different *sympatric* populations could reduce gene exchange between them (Hoskin et al. 2005).

Alternatively, heterospecifics might be distributed across space as a gradient, so that sympatry transitions into allopatry as heterospecifics become less common (Hopkins et al. 2014). If the extent of divergence in mating traits between incipient species depends on heterospecific frequency, then each sympatric population along a gradient could have a different distribution of mating traits (Waage 1979; Cooley 2007; Kuitunen et al. 2011; Hopkins et al. 2014). Such a frequency-dependent response to heterospecifics is especially likely if the mating traits undergoing reinforcement are subject to countervailing patterns of selection (Pfennig 1998; e.g., Pfennig 2000; Hopkins et al. 2014; Hopkins and Rausher 2014). Whether reproductive isolation arises between allopatric populations and any given sympatric population (or among sympatric populations) would depend on dispersal distances and how they corresponded to mating trait divergence.

A third ecological factor that impacts the potential for reinforcement to promote species proliferation is community composition. Although reinforcement and its downstream effects are typically considered in light of pair-wise interactions (Jaenike et al. 2006; Porretta and Urbanelli 2012; Bewick and Dyer 2014; Hopkins and Rausher 2014; Pfennig and Rice 2014; Kozak et al. 2015), species often occur with different species across their range, and pair-wise interactions occur within different community assemblages (McPeek and Gavrilets 2006; Pfennig and Ryan 2006; Muchhala and Potts 2007; Lemmon 2009; Hoskin and Higgie 2010; Crampton et al. 2011). Because reinforcement with different species can favor the evolution of different traits (Pfennig and Ryan 2007), different sympatric populations will diverge not only from allopatry but also from each other, and species proliferation can result (McPeek and Gavrilets 2006; Pfennig and Ryan 2006). Such species proliferation is possibly more likely in the context of adaptive radiations where a given species might experience deleterious reproductive interactions with different heterospecifics across its range.

A fourth ecological factor that impacts whether reinforcement generates species proliferation is environmental variation that underlies selection in sympatry and allopatry. By definition, reinforcement occurs under conditions where hybridization between species or incipient species is selectively disfavored. Yet, a feature that emerges from recent analyses of adaptive radiations is that hybridization might play an important role in the transfer of genetic variation that generates novel traits and enables the invasion of—and rapid adaptation to—novel environments or niches (Seehausen 2004; Grant et al. 2005; Mallet 2009). Such hybridization has the potential to break down the process of species proliferation by impacting whether reinforcement occurs, or, if reinforcement does occur, whether it remains stable over time. In particular, whether reinforcement occurs and the extent to which it does so depends on whether or not hybridization is fitness enhancing. However, the fitness consequences of hybridization can vary with the environment (Parris 2001; Fitzpatrick and Shaffer 2004; Craig et al. 2007; Pfennig 2007; Campbell et al. 2008), vary between the interacting species (Pfennig and Simovich 2002), or vary over time if deleterious genetic interactions are removed by selection or if modifiers of genetic incompatibilities evolve

(Barton and Hewitt 1985, 1989; Sanderson 1989; Ritchie et al. 1992; Lammers et al. 2013; Schilthuizen and Lammers 2013; Seidl et al. 2019). Such variation is more likely between closely related species, in which the potential for beneficial hybridization is greater than between distantly related species.

Regardless of how variation in hybrid fitness arises, the potential for hybridization to be beneficial under even some circumstances could limit reinforcement (Liou and Price 1994) and therefore, the potential for subsequent species proliferation. Moreover, if hybrids are less fit in some environments than in others, then the extent to which reinforcement unfolds could vary across sympatry (Mandeville et al. 2015). Similarly, if one species experiences selection to avoid hybridization (and undergoes reinforcement) but the other species does not (Cooley 2007), then proliferation might occur only in the lineage that underwent reinforcement. Indeed, such a contrast can provide an opportunity to evaluate reinforcement's role in species proliferation.

Finally, environmental heterogeneity will not only impact selection against hybridization, but it can also impact the nature of selection on mating traits for reasons other than the avoidance of hybridization. Indeed, mating trait evolution is often shaped by the environment (Andersson 1994; Endler and Basolo 1998; Boughman 2002; Rosenthal 2017). During adaptive radiations, in particular, mating traits can evolve as indicators of local adaptation or in correlation with locally adapted ecological traits (Podos 2001; Huber and Podos 2006; Boughman 2007; Van Doorn et al. 2009; Kelly et al. 2019). Adaptive radiations are contexts where environmental heterogeneity—and local adaptation—could enhance the likelihood that reinforcement between species in sympatry promotes downstream divergence within species, if the underlying environmental variation promotes opposing selective patterns between sympatry and allopatry.

Behavioral Factors Impacting the Likelihood that Reinforcement Leads to Species Proliferation

When reinforcement leads to divergence in mating traits (such as preferences and signals) among conspecific populations, this divergence has the *potential* to cause reproductive isolation. Additional behaviors—such as mate sampling and choosiness—modulate how mating traits are translated into actual reproductive decisions (Real 1990; Kozak et al. 2013; Tinghitella et al. 2013; Rosenthal 2017), and they can thereby determine the extent to which these diverged mating traits generate reproductive isolation between sympatric and allopatric populations. Although such factors are well known for impacting reproduction, it is not clear what role they play in reinforcement's downstream effects on species proliferation, if any.

How prospective mates are assessed might have as important of an effect on reproductive isolation between sympatric and allopatric populations as the extent of divergence in mating preferences or sexual signals. Generally, reproductive isolation might be less likely if individuals accept any mate that meets a minimum threshold (Janetos 1980). For example, if individuals in an allopatric population possess such a threshold, they will mate with any immigrant derived from a sympatric population that possesses a trait above the threshold, even if the immigrant is less attractive than the average allopatric resident. Instead, reproductive isolation between populations might be more likely if individuals compare many prospective mates before mating (a so-called "best-of-n" strategy; Janetos 1980), because migrants (and their signal phenotypes) will be less attractive

than the residents to which they are being compared, especially if immigrants are rare (McPeek and Gavrilets 2006).

Moreover, whether reproductive isolation occurs even when mating traits are divergent depends on the willingness of individuals to court or mate with those that do not possess preferred traits (i.e., "choosiness"; Rosenthal 2017). Choosiness can vary among individuals within a population depending on life stage (e.g., whether they are previously unmated or aging) or opportunities for additional matings (Backwell and Passmore 1996; Gabor and Halliday 1997; Moore and Moore 2001), and among populations depending on encounter rates with prospective mates (Berglund 1995; Willis et al. 2011) or the risks and costs of mate assessment (Forsgren 1992; Berglund 1993; Willis et al. 2012). If choosiness is high (i.e., individuals are unwilling to mate with unattractive individuals), reproductive isolation is more likely than in systems where choosiness is low (i.e., individuals are willing to mate with unattractive individuals). Indeed, when choosiness is low, the distribution of individuals who mate successfully would be wider than predicted from laboratory measures of mate preferences. In other words, behavior tests can over-estimate reproductive isolation in nature. Because many ecological factors (e.g., predation risk, population density, sensory environment) and evolutionary factors (mating system, sex ratio, sensory system) affect choosiness and expression of mating preferences and signals (Andersson 1994; Rosenthal 2017), such factors should be considered in evaluating whether reinforcement in sympatric populations generates downstream reproductive isolation between conspecific populations in sympatry and allopatry.

Evolutionary Factors Impacting the Likelihood That Reinforcement Leads to Species Proliferation

Given that reinforcement generates divergence in mating traits in sympatry, 2 evolutionary factors will enhance the likelihood of downstream reproductive isolation between sympatric and allopatric populations and species proliferation: 1) the extent to which divergence in mating traits that contribute to isolation coincide in the sexes (e.g., mate preferences versus sexual signals might not diverge to the same extent in sympatry versus allopatry); and 2) the evolutionary stability of reinforcement, and, concomitantly, the evolutionary persistence of traits that evolved via reinforcement.

Reinforcement can impact mating traits that are the same in both sexes (known as "matching" traits: Kopp et al. 2018), such as, for example, timing or location of mating (Via 1999; Silvertown et al. 2005). Migrants of either sex could therefore be at a disadvantage if such traits diverge between populations in sympatry versus allopatry with reproductive isolation arising as a result. Alternatively, reinforcement can impact traits that are exhibited in only one sex, such as, for example, female mate choice or male sexual signals used to court females. However, divergence in sympatry between incipient species does not necessarily occur to the same extent in these different types of traits (Howard 1993; Höbel and Gerhardt 2003; Kozak et al. 2015). In other words, traits possessed by females might diverge more (or less) than traits possessed by males in sympatry relative to allopatry. That males and females might differ in the extent to which their traits diverge will thereby impact the likelihood of assortative mating by population type. Specifically, if reinforcement in sympatric populations causes divergence from allopatric populations in traits expressed in only one sex, then gene flow could still occur between sympatric and allopatric populations. For example, if male traits diverge between sympatric and allopatric

populations, but female mate preferences do not, then males—but not females—that migrate to a different population type would be selectively disfavored. Thus, species proliferation is most likely when reinforcement causes divergence in the mating traits of both sexes between sympatric and allopatric populations.

Additionally, reinforcement is more likely to generate downstream reproductive isolation between sympatric and allopatric populations if reinforcement is stable within sympatric populations over time. If, however, reinforcement breaks down or varies over time, then mating traits in sympatric populations might not remain divergent from those in allopatric populations. Consequently, any reproductive isolation between sympatric and allopatric populations would also break down. Whether reinforcement is stable depends, in part, on the costs of hybridization, which can vary in space or time (as discussed above). The breakdown of reinforcement could explain why adaptive radiations do not occur or are limited in their diversity.

Empirically Evaluating Reinforcement's Role in Species Proliferation and Adaptive Radiation

Evaluating reinforcement's role in species proliferation requires identifying whether reinforcement in sympatry generates downstream reproductive isolation among conspecific populations. Yet, identifying whether reinforcement has occurred in the first place is nontrivial, and a large literature exists on how to ascertain whether it might have occurred (Butlin 1987; Howard 1993; Servedio and Noor 2003; Coyne and Orr 2004; Pfennig and Pfennig 2012). Many of the same difficulties with demonstrating reinforcement also apply to evaluating whether reinforcement in sympatric populations instigates reproductive isolation between sympatric and allopatric populations. Below, we suggest different empirical approaches to this problem.

Finding reductions in both mating and gene flow between sympatric and allopatric populations is consistent with the hypothesis that reinforcement between species in sympatric populations can initiate reproductive isolation between sympatric and allopatric populations (Figure 1). As described above, evaluating mating success of migrants and residents in nature, or measuring gene flow among conspecific populations (Pfennig and Rice 2014; Lemmon and Juenger 2017) is stronger evidence regarding reproductive isolation than lab-based mate preference assays. Moreover, alternative explanations to reinforcement for reproductive isolation among conspecific populations should be tested, as described below.

If mating and gene flow are reduced between sympatric and allopatric conspecific populations, the key question is whether reinforcement in sympatry has *caused* the observed patterns of reproductive isolation. For example, reproductive isolation between sympatry and allopatry could arise because they are ecologically different, and ecological selection against migrants or dispersal barriers have reduced gene flow (Noor 1999; Rundle and Nosil 2005; Ortiz-Barrientos et al. 2009; Nosil 2012). Reinforcement could therefore promote mating trait divergence that is co-incident with, rather than causal of, reduced gene flow between sympatry and allopatry, and disentangling these alternative scenarios is important.

Before describing approaches for doing so, we first raise a key caveat. Namely, although reinforcement, ecological selection, and limits to dispersal are often treated as mutually exclusive or alternative explanations for reproductive isolation, it is important to emphasize that they can operate in tandem to generate reproductive isolation (Nosil et al. 2003; Rundle and Nosil 2005; Nosil et al. 2007; Ortiz-Barrientos et al. 2009; Nosil 2012). Indeed, the confluence of these factors is likely during adaptive radiations. Thus, finding that ecological selection or dispersal barriers are associated with patterns of gene flow among conspecific populations does *not* rule out reinforcement as a cause of reproductive isolation. Nevertheless, the effects of each potential agent of reproductive isolation should be explicitly evaluated (either statistically or through choice of experimental design).

One approach to disentangling the different effects of ecological selection and reinforcement on reproductive isolation is to leverage variation among multiple sympatric and allopatric populations (Higgie and Blows 2007; Nosil et al. 2007). Statistical modeling could then be used to ascertain the extent to which mating traits, ecological factors, or dispersal explain reproductive isolation among populations (see also Boughman et al. 2005). If reinforcement generates downstream reproductive isolation, then reproductive interactions with heterospecifics should better predict mating traits and gene flow across conspecific populations than putative ecological factors or dispersal barriers that also could cause reproductive isolation among conspecific populations (Nosil et al. 2003, 2007; Pfennig and Rice 2014; Lemmon and Juenger 2017).

Additionally, the extent of reproductive isolation between sympatric and allopatric populations could be related to variation in the location or strength of reinforcement across time (sensu Boughman et al. 2005). In particular, as reinforcement unfolds in sympatric populations, reproductive isolation should also accumulate between sympatric and allopatric populations. Historical data, such as genetic and trait data in museum collections or long-term data (Grant and Grant 2008; Bi et al. 2013; Guschanski et al. 2013), could be used to evaluate whether reproductive isolation between sympatry and allopatry is correlated with the unfolding of reinforcement in sympatry. Moreover, phylogenetic and phylogeographic analyses, in which population history and relationships among populations are used to infer historical patterns and evolutionary relationships, can be combined with these approaches to evaluate whether reinforcement generates downstream species proliferation as expected. For example, if reinforcement generates reproductive isolation, then sympatric populations (and the traits expressed) should be derived from allopatry, not vice versa. In other words, allopatry should *not* consist of populations that were established by dispersers out of populations that already underwent reinforcement.

In addition to these above approaches, experimental work could test key predictions of the hypothesis that reinforcement's downstream effects promote species proliferation. Such approaches could include the generation of artificial populations to disentangle the effects of mating versus ecological traits (sensu Stelkens et al. 2008) or experimental evolution to evaluate whether reinforcement generates downstream isolation between sympatry and allopatry (sensu Rice and Salt 1988).

Finally, a comparative approach is a further means for evaluating whether reinforcement leads to species proliferation (sensu Coyne and Orr 1989; Seehausen and Van Alphen 1999; Arnegard et al. 2010; Wagner et al. 2012). In particular, rates or extent of species proliferation could be compared among adaptive radiations (e.g., on an island archipelago) that vary in likelihood of reinforcement-initiated species proliferation. In particular, we predict that radiations that were initiated by the multiple closely-related colonizers (e.g., sister species or congeners) would undergo greater species proliferation than those that were initiated by a single colonizer or by lineages that were already so diverged in mating traits as to not undergo reinforcement. Moreover, ancestral character state reconstruction of

mating traits could reveal whether similarity in mating traits among species early in the radiation predicts the amount of subsequent proliferation. One issue to consider, however, is whether extinction or fusion of the hybridizing taxa in sympatry could potentially alter the perceived frequency with which reinforcement and its downstream consequences occur (Templeton 1981; Coyne and Orr 1989). Yet, because extinction or fusion should reduce diversity as a consequence of sympatry, it can be contrasted with the hypothesis that reinforcement promotes diversity.

Conclusions

Ecological opportunity fosters adaptive divergence among populations of a founding lineage and can thereby explain species proliferation during adaptive radiations (Losos and Mahler 2010; Yoder et al. 2010; Stroud and Losos 2016). Reinforcement is both a likely consequence of this divergence, and a potential agent of further divergence. Indeed, the ecological and evolutionary conditions of adaptive radiations are particularly conducive to reinforcement *among* radiating lineages, and, as highlighted herein, reinforcement's diversifying effects on mating traits *within* lineages can initiate further species proliferation (Figure 1). Thus, reinforcement is predicted to facilitate species proliferation during adaptive radiations.

Whether this happens depends on the overall landscape of sexual selection in which reinforcement is operating: if reinforcement causes mating traits to evolve that are maladaptive in other conspecific populations (e.g., allopatric populations, or sympatric populations where reinforcement proceeds differently), the resulting patterns of opposing mating traits could generate reproductive isolation among conspecific populations. Reinforcement-initiated species proliferation is expected to be greatest if: incipient species are distributed patchily; community composition is variable; and ecological, behavioral and evolutionary factors generate divergent mating patterns that actually reduce gene flow among populations. These impacts of reinforcement have not been widely evaluated, but adaptive radiations might be particularly well suited for discerning reinforcement's role in diversification. Because the ecological and evolutionary contexts in which adaptive radiations often occur make reinforcement likely, reinforcement might play a crucial, but underappreciated role in determining the nature and extent of diversity that arises—and persists—in adaptive radiation.

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