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Reply to Andrew Brower's critique of the evidence for hybridization among *Heliconius* butterfly species in the wild

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Abstract

Andrew Brower recently published a long article in this journal that seeks to dismantle evidence for hybridization between species of *Heliconius* butterflies. The main evidence that Brower criticizes here is given in two papers published by my colleagues and myself in 2007. In this reply, I briefly defend our evidence, and at greater length provide additional background information to help establish the credibility of the evidence even more firmly than previously.

Key words:

Introduction

Andrew van Zandt Brower recently published a paper (Brower 2018a) arguing that many interspecific hybrid specimens we reported in our review of hybrids among species of *Heliconius* (Mallet *et al.* 2007) were unreliably identified. He further argues that a wild hybrid between *H. ethilla* and *H. melpomen*e, captured by our team in Peru and verified as a hybrid using DNA sequencing (Dasmahapatra *et al.* 2007), is also open to doubt. The combined studies of many colleagues and myself, both then and since, has shown that hybridization among many *Heliconius* species occurs at a low rate in nature, and also that introgression of genetic material is a somewhat frequent result of these occasional misalliances. Previously, Brower issued various other critiques of our body of work on hybridization and gene flow among *Heliconius*, but we had not so far taken these very seriously. I defended our work in two blog posts in January 2013 (eratosignis 2013a;b).

The most recent paper (Brower 2018a), however, is unusual for the very detailed nature of his attempts to cast doubt on our work. Firstly, the paper is an 87-page monograph that goes through all the supposedly incorrect assessments perpetrated by Mallet *et al.* (2007), specimen by specimen, as well as denying additional evidence published since. Secondly, Brower's title is "Alternative facts: a reconsideration of putatively natural interspecific hybrid specimens in the genus *Heliconius* ..." In essence, this title appears to accuse my colleagues and myself of falsification.

The meaning of "alternative facts"

Although "alternative facts" is a generally understood meme today in some parts of the world, it is worth outlining here for international readers that the term is a neologism referring to Presidential Advisor Kellyanne Conway's 2017 defence of Sean Spicer, the Press Secretary of the President of a well-known republic. The President had asserted in a televised speech in the CIA on 21 Jan 2017, the day after his inauguration, that unprecedented crowds had welcomed him at the inauguration: "I made a speech, I looked out, the field looked like ... a million, a million and a half people. ... it went all the way back to the Washington Monument". Spicer argued that media reports to the contrary were incorrect, and that "this was the largest audience to ever witness an inauguration -- period -- both in person and around the globe."

The problem for the story given out by the President and Spicer, immediately pointed out by the press, was that the National Mall had been photographed from the air during the inauguration. In fact, the crowds seemed rather

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exiguous compared to similar photos of the crowds that had greeted the previous President's inauguration in 2009. It was hard to accept the President's, or Spicer's assertions, when all you had to do was check the images and compare them with those of the inauguration of 2009. Kellyanne Conway in an interview on 22 Jan 2017 attempted to defend Spicer to an incredulous interviewer by saying "Sean Spicer gave alternative facts". These alternative facts seemed to be falsehoods, unless you believed the President and his people more than you believed your own eyes. The Washington Post's Fact Checker shows that the same President delivered around 10,000 "false or misleading claims" in only 800 days of office. Brower, via his jokey title, seems to compare my colleagues and myself to one of the best documented liars in history.

What this paper aims to do

To support his skeptical view of hybridization in *Heliconius*, Brower (2018a) needed to counter our conclusions in excruciating detail, specimen by specimen, simply because we had provided such a wealth of evidence in the form of images of these putative hybrids, from many different countries and collectors from across the neotropics, in a form freely available to readers, and because we documented in detail the reasoning behind our decisions on the specimens.

We finally published our paper (Mallet et al. 2007) during what were still the early days of online-only journals, at the start-up publisher BMC (more recently, taken over by Springer). Unlike more traditional journals of that time, BMC had the enormous advantage of providing open access content purely online, so we were able to marshal all the evidence and photos we could find on the hybrids, and we submitted these as an HTML database of photos in an extensive online appendix. The photos were used so that even if you didn't believe our words, you could see almost all the evidence that backed up the paper's conclusion with your own eyes.

I have checked all Brower's re-interpretations of our documented hybrid specimens in his critique. Apart from a few minor errors (for instance the identity of the subspecies of some of the parent species), I stand by the majority of our initial diagnoses. If you lack knowledge of these specimens, I think I am unlikely to convince you, the general reader, and I am even less likely to convince Brower, by simply going through the evidence all over again, specimen by specimen. And of course, we had used no molecular data for our inferences, which were based purely on morphology and our experience of the genetics of interspecific and intraspecific crosses of some of the species (Mallet et al. 2007). Our original decisions were well-documented and based on many consultations among the authors and with outside parties. They were our best attempts at inferring the hybrid status of these hybrids at this time. Brower is as far as I know unique among those who have studied *Heliconius* extensively in denying that such specimens represent real hybrids between species occurring in the wild. This may partly reflect his lack of knowledge of the species in the field (his main field work, as far as I understand it, was in Central and South America mostly during his graduate student days) and a lack of experience of making laboratory crosses with these species. In contrast, I and my own early mentors Keith S. Brown Jr., Lawrence E. Gilbert, John R. G. Turner, and Gerardo Lamas Müller, as well as my more recent colleagues, such as Owen McMillan, Chris Jiggins and Mathieu Joron, and their students and postdocs, all have much more field and/or lab crossing experience than Brower and accept most of these specimens to be hybrids. In many cases, in our paper, we simply accepted the authority of earlier identifications of the specimens as hybrids published by others.

While it seems pointless to go through each specimen again, I should note that with today's molecular genetic technology, it would be possible to test any of our earlier assertions by sequencing tiny fragments of even quite old museum specimens, if Brower or anyone else seriously seeks to prove that these specimens are not hybrids.

However, Brower also casts doubt on the provenance of some of these specimens, particularly of the many hybrids collected in Colombia and Venezuela, apparently believing that even if they are hybrids, they were "manufactured" in insectaries as a means of enhancing commercial value on the international market for butterfly specimens. In this paper I divulge hitherto unpublished background information about some of the collectors and collections that I believe attests to much greater reliability of the provenance of these specimens than Brower allows, and I document our extensive field work and other checks to verify the hybrid specimens we cited.

Finally, on both technical and provenance grounds, Brower criticizes our DNA analysis (Dasmahapatra *et al.* 2007) of a field caught hybrid collected by my own group. Our original publication was very telegraphic, so I here expand on the details given there, in order to clarify the support for our interpretation and to counter Brower's criticisms.

How did Mallet *et al.* (2007) come to be written?

In the late 1970s to early 1980s, I had studied hybrid zones between divergently coloured races of *Heliconius erato*, and also between the matching Müllerian co-mimic subspecies of *H. melpomene*, in Panama, Colombia, and Peru. With Chris Jiggins and Owen McMillan, in the late 1980s and early 1990s I started a new project in Southern Ecuador on a hybrid zone between *Heliconius erato cyrbia* and *Heliconius himera*. Previously, *H. himera* had been classified as yet another local subspecies of *H. erato* differing in colour pattern from the rest of the species. Following up pioneering work (Descimon & Mast de Maeght 1984), we documented this Ecuador hybrid zone in some detail in a series of experimental, molecular genetic, and observational papers (Jiggins *et al.* 1996; Jiggins *et al.* 1997b; Jiggins *et al.* 1997a; Jiggins & McMillan 1997; McMillan *et al.* 1997; Mallet *et al.* 1998a; Davison *et al.* 1999).

In the centre of the narrow overlap zone, ~5% of "pure" *H. erato* and *H. himera* individuals mate naturally with the other species in the centre of the hybrid zone (Mallet *et al.* 1998a). We concluded that, in view of the paucity of intermediates due to natural hybridization in zones of overlap, the two taxa should be considered separate species. Nonetheless, the relatively high rate of natural hybridization in their overlap zone clearly represented a somewhat intermediate stage between species as usually conceived of at that time, and subspecies. The *H. himera* x *H. erato* studies, together with related work on gene flow between ecological host races of insect herbivores (Emelianov *et al.* 1995; Emelianov *et al.* 2004; Drès & Mallet 2002), suggested to us that "reproductive isolation" formed an approximate continuum across the species boundary. Although species eventually acquire some degree of reproductive isolation, the lack of useful absolute criteria from reproductive isolation led me to a view that species, if they hybridize with other species, were better viewed as recognizable "genotypic clusters" that can maintain multilocus differences in the face of gene flow (Mallet 1995), rather than as reproductively isolated populations, as in the then widely applied "biological species concept".

However, the focus on reproductive isolation generated by the mid-20th Century biological species concept had led to relatively little interest in amassing data on which species hybridized – animal hybrids were acknowledged to exist occasionally in nature but were considered both trivially rare and unimportant evolutionarily. One exception I knew of was a book in Russian by E.N. Panov, who showed that around 9% of the world's bird species were known to hybridize; Panov's data had been recently summarized (Grant & Grant 1992). The Grants in the same article also documented extensive hybridization among Darwin's finches on the Galapagos.

I had long worked with *Heliconius* butterflies from South and Central America and had been aware from literature records and personal knowledge of major collections from around the world that a number of putative interspecific *Heliconius* hybrid specimens existed. By 1995 I was starting to collect a list of these wild-caught hybrids and photographs of them with data from a variety of collections. In a minor part of a book chapter, we published the first table of all the hybrid specimens I then knew of, estimating that around 25-28% of species hybridized in nature (Mallet *et al.* 1998b). The identity of the hybrids was based largely back then on the opinions of other experts, particularly Keith S. Brown (Campinas, Brazil), Phillip Ackery and Robert Smiles, who had worked with Keith Brown on the BMNH *Heliconius* type specimens in London (Ackery & Smiles 1976), as well as Helmut Holzinger (Vienna), my own PhD supervisor Lawrence E. Gilbert (University of Texas, Austin), and Walter Neukirchen (Berlin).

Around 1996, I conceived of a plan to review all known wild-caught hybrid *Heliconius* specimens in some detail, and soon thereafter began to develop a public database at UCL, with *Heliconius* hybrids listed along with photos of upper and undersides. By 1997 these totalled 56 hybrids (Mallet & Neukirchen 1997). In 2001 we submitted a manuscript to Systematic Biology outlining the results from an expanded database with 136 wild-caught hybrids.

The manuscript was rejected, in part because at that time the editorial team at Systematic Biology and reviewers doubted that this admittedly rather observational study of rates of hybridization was a suitable topic for a systematics journal. One of the critical reviewers signed his name: Andrew Brower. The reviewers' comments were, however, very helpful in improving the paper, which was finally published with additional data in the open access online journal, BMC Evolutionary Biology (Mallet *et al.* 2007), resulting in the published paper that, 11 years later, Brower found so controversial.

The number of putative interspecific hybrids now totalled 161 (of which 7 were in the sister genus *Eueides*, and 154 in *Heliconius*). The fraction of species of Heliconiini hybridizing in the wild was now estimated as 26-29%; in the genus *Heliconius*, 33-35% of species hybridized (Mallet *et al.* 2007). I emphasize (and we did then also) that hybridization is not a common phenomenon in nature. Apart from the *H. erato x H. himera* hybrids, our best guess is

that it is very rare on a per individual basis. For the next best studied case, *H. cydno x H. melpomene*, we estimated only around 0.05% of individuals collected in areas of overlap are hybrids (Mallet *et al.* 2007). But collectors have always been attracted to rarities, and closely related *Heliconius* species are often divergently coloured, so that hybrids are conspicuous and tend to find their way into museum collections. Thus, *Heliconius* provides a particularly useful genus for discovery of any rare hybrids that do occur in nature.

Brower's conclusions from his reanalysis of the specimens

In order to counter our extensive evidence, Brower develops his own "reliability score" for each hybrid specimen, obtained by multiplying scores based on his personal answers to five different questions: (1) Is the hybrid interspecific? (2) Were the putative parental species correctly identified (or are they identifiable)? (3) Is the specimen's wing pattern demonstrably "interspecific"...? (4) Are the hybrid's locality data plausible? (5) Are the specimen's provenance and chain of custody reliable? The alert reader might notice that there is some reduplication of ideas among these questions, for example between 1,2, and 3, or 4 and 5, suggesting that a considerable level of double jeopardy has been built into Brower's reliability scores. He continues:

> Questions 1–3 are [then] combined into a single identity score, ranging from 0–1, with 1 being certainty that the specimen is indeed an interspecific hybrid. Questions 4 and 5, relating to quality and plausibility of the locality data and other circumstances of the specimen's acquisition are combined into a second authenticity score. The product of these two scores gives a quantitative, albeit subjective, measure of the specimen's relative reliability.

Armed with these reliability estimates, he is then able considerably to reduce the "problem" of hybridization in *Heliconius*. Around 65 specimens (including some discovered since 2007 and not on our database) received a score of 10% reliability or less, enabling him immediately to exclude almost half the specimens from consideration! He subsequently concentrates on "reliable" specimens with scores of 50% or more, by his criteria (e.g. in his Table 1).

"Who are you going to believe? Me? Or your own eyes?" (Chico Marx)

I have now carefully checked each of Brower's republications of our 2007 figures, to which Brower has helpfully added images of putative parental taxa (something we did not do and perhaps should have done in the original paper).

Now that we have studied *H. elevatus* and *H. pardalinus* in more detail (Rosser *et al.* 2019), I concede that it is possible that some, but not all of the hybrids that we had inferred were between silvaniform *Heliconius* (the ithomiine-mimicking subgroup) and "dennis-rayed" Amazonian races of *H. melpomene* (hybrid nos. 8–13) may actually represent hybrids between those silvaniforms and *H. elevatus*. *Heliconius elevatus* is a unique silvaniform with a *melpomene*-like rayed pattern (well-illustrated in Brower's article). I should here mention that recent genomic evidence argues that *H. elevatus* owes its unique *H. melpomene*-like rayed pattern to colour pattern-determining regions of the genome that introgressed between *H. melpomene* and the ancestor of *H. elevatus* (*Heliconius* Genome Consortium 2012; Wallbank *et al.* 2016). *Heliconius elevatus* therefore appears to be a hybrid species now sympatric with the descendants of both of its parents, unlike *H. heurippa*, which was hypothesised to be a hybrid species, but is sympatric only with one parent, *H. melpomene* (Mavárez *et al.* 2006). Thus although Brower argues that *H. elevatus* might be more likely to hybridize with silvaniforms than with *H. melpomene* (an argument with which I concur), it is ironic that *H. elevatus* is itself the product of the latter kind of union.

Our arguments for hybrids between silvaniforms and the red-banded "postman" *H. melpomene* subspecies (hybrid nos. 17–20), on the other hand, are all correct, I believe, or at least I'd put their reliability at >> 0.75 by using my own reliability score, backed up in most cases by other experts, such as published work by Keith S. Brown.

Apart from this caveat about *H. elevatus* hybrids, I stand by all of the rest of our original decisions, which were well-documented and were our best attempts at identification of these putative hybrids at the time. Although it would be fruitless to go through each specimen again, it may be worth outlining two general areas where Brower's

low assessments of reliability seem to me particularly erroneous. The first is about specimens originally collected by amateurs or commercial collectors, particularly in Colombia and Venezuela, which Brower apparently finds unreliable, especially in his assessments based on questions 4 and 5 about provenance. The second concerns Brower's assertions about our DNA analysis of a hybrid specimen between *H. ethilla* (a silvaniform) and a red-banded "postman" *H. melpomene*, captured in the field by members of my own research group. It seems important for his argument for Brower to discount hybridization as distant as this, and he seems to expend some effort to ensure that his reliability score for this specimen is below his 0.75 cut-off.

Putative hybrid specimens collected by amateurs and commercial collectors in Colombia

Many of the hybrids we cited do in fact come from the Magdalena valley and nearby regions of Colombia, and Brower uses this fact to cast doubt on most hybrids from this region. And, to be fair, the abundance of hybrids from Colombia still seems somewhat remarkable to me, as well. Their abundance may in part result from the fact that many butterfly specimens in general come from this area. Butterfly collecting has a very long history in the Colombian rainforest, especially in the "zona esmeraldífera" near Muzo and Otanche, Boyacá where mining for gems led to 19th and early 20th century colonization of inter-Andean foothill rainforests normally rather inhospitable to European-style agriculture, and while the navigable Magdalena River was still the most important trade route between Caribbean ports and the capital, Bogotá. Today, some butterfly collectors may indeed be breeding butterfly hybrids for the market (and in 2007 we excluded certain suspect hybrid specimens for this reason, as discussed in the paper). However, commercial breeding was certainly not carried out in Colombia before the 1980s, when Clive Farrell first set up his London Butterfly House at Syon Park. This was as far as I know the first commercial butterfly greenhouse that required a supply of bred livestock from the neotropics.

A number of our hybrids originated from Otanche, and so to investigate this, my Colombian co-author Dr. Mauricio Linares, at that time Professor of Genetics at the Universidad de los Andes, and I visited in Otanche for several days in 1997. The city was then under the control of paramilitaries, essentially a series of local mafias that had been legitimized by the Colombian government in an attempt to stabilize regions affected by guerilla activity. We were helpfully advised when we checked in at the hostal that we should quickly obtain permission for our visit from the paramilitary "capo." We duly presented ourselves at his house, and found he was a paraplegic in a wheelchair, reportedly due to a spinal injury sustained during a past gun-battle. Luckily for us, he welcomed us to Otanche, perhaps because he too had an interest in butterflies. He had a large display of pinned iridescent blue *Morpho cypris* butterflies on his wall, and indeed, while revenues from the spent emerald mines were dwindling, butterfly specimen exports perhaps seemed to hold financial promise for the region. We were able to visit a prominent local collector, José Urbina, who had exported many butterfly specimens from Otanche, including a few of the interspecific hybrids we listed in 2007; he was an old-fashioned commercial collector and did not have insectary facilities. In contrast, Brower, lacking any personal knowledge of this collector, concludes without evidence that all Urbina's specimens have very low authenticity because he had a commercial interest in them.

Nonetheless, I agree with Brower that the recent possibility of the commercial production of hybrids in insectaries adds to the importance of knowing something about the collector and their collections. One such prolific collector, who had a number of key *Heliconius* hybrids from Colombia that we cited, was Dr. Ernesto Schmidt-Mumm, an optician and amateur lepidopterist. I had known him since being invited to supper at his Bogotá apartment, in 1977. Dr. Schmidt-Mumm related to me then how he would go out collecting with his dog, which would disturb butterflies, and he claimed his dog aided his catch. He never reared any butterflies from caterpillars. After Schmidt-Mumm's death in August 2000, his important collection was accessioned by the Instituto Humboldt, Villa de Leyva, Boyacá, on 12 May 2001 (Salazar 2002). Andrew Brower, citing a personal communication from Keith Willmott, alleges that the locality labels on these specimens are unreliable, and via his questions 4 and 5 about provenance casts doubt on many of Schmidt-Mumm's interspecific hybrid specimens.

However, what Brower and Willmott perhaps do not know is that both Mauricio Linares and I took notes on Schmidt-Mumm's collections at his house in Bogotá from 1977 onwards, while he was still actively collecting, and long before his collections were in the Instituto Humboldt. I have known about these hybrid specimens for over 40 years, and Keith Brown had also visited and documented the *Heliconius* hybrids in the same collection before me. Schmidt-Mumm was an amateur, but in contrast to Brower's assessment, I rate Schmidt-Mumm's collections very

reliable. He had a ranch on the Río Negro near Villavicencio, Meta. Some of the disputed specimens were collected in this area, on the road between Bogotá and Villavicencio that runs along the Río Negro valley, an area I visited repeatedly (Mallet & Jackson 1980). Schmidt-Mumm also owned a farm in Victoria, Caldas, in the Magdalena Valley, where still other hybrid specimens were collected. Keith Brown visited Schmidt-Mumm at his Victoria estate and reports capturing a *H. cydno* x *H. melpomene* hybrid himself at Victoria on 21 Jan 1971 (Brown & Mielke 1972, p. 10).

Schmidt-Mumm pinned and spread his own specimens, but he had an idiosyncratic method of data labelling. He used double-sided insect boxes, with the butterflies pinned on the bottom of the box, and the data label pinned in the corresponding position on the cork lid of the same box. As well as being in a position that corresponded to the specimen in the lower drawer, the labels also bore Dr. Schmidt-Mumm's identification to species and in some cases to subspecies, so there was never much doubt which label referred to which specimen in Schmidt-Mumm's original boxes. To professional entomologists like Willmott and Brower, separating the label data from the specimen does indeed seem very bad practice!

However, Schmidt-Mumm knew his specimens well, and I didn't notice any mislabellings among the *Heliconius* when I saw and photographed some of the specimens in the 1970s. In the 1990s, Mauricio Linares also photographed the original storage boxes with key hybrid specimens at Schmidt-Mumm's home, and also the corresponding labels from the lids of the boxes. I have in my possession the original Kodak transparencies sent to me by Mauricio Linares during the gestation period of our original 2001 manuscript on *Heliconius* hybrids. Matthias Nuss, from the Senkenberg Museum in Dresden, had also visited Ernesto Schmidt-Mumm's house and supplied excellent photos of these same boxes to Walter Neukirchen in the 1990s. Our 2007 data from this collection were based on these original storage box photos and the data labels that had been hand-written by Dr. Schmidt-Mumm. We did not depend on the newer printed data labels cited by Brower that the specimens now bear at the Instituto Humboldt; as Willmott notes, these might potentially have led to mix-ups. Wherever possible, however, we did use Jean-François Le Crom's more recent single specimen photos of Schmidt-Mumm's hybrids from the Instituto Humboldt for later versions of our online database because Le Crom's photos were of much higher quality than our own earlier images. Our earlier images were based on digitally extracting specimen images from transparencies of whole boxes of specimens from Schmidt-Mumm's home (for examples of some earlier images that we were unable to replace, see hybrid nos. 19 -upperside - and 53).

A case in point is the uniquely aberrant specimen we designate as a probable *H. elevatus* x *H. hecale* hybrid (specimen no. 14, Brower's Fig. 21). By a variety of different arguments, in part about "authenticity," Brower assigns this specimen a low overall reliability of 0.15. However, I can simply return to our original transparency, and decipher Schmidt-Mumm's handwritten label pinned the lid of the box as follows:

> *Heliconius melpomene bari* Oberth.
> + *numata* [this latter is in later writing, possibly by Keith Brown]
> Puerto Inírida
> 16 IV 74

Since Schmidt-Mumm had no other specimens that look remotely similar to *Heliconia bari* Oberthür, the label, whose position in the lid corresponded to the position of the specimen in the bottom drawer, clearly refers to this very specimen. (*Heliconia bari* is today considered a subspecies of *H. elevatus*, but was apparently believed by Schmidt-Mumm to be a subspecies of *H. melpomene*). In contrast, Brower, lacking our background knowledge, expresses his doubts by first citing a personal communication from Keith Willmott as follows (bold is my emphasis):

> "I have to say this looks like a pretty unusual '*elevatus*' variant, and **some kind of hybrid origin seems perhaps more likely**, but since I would say the label data are highly unreliable, we essentially don't know where it was collected at all. So, difficult to speculate further about a hybrid origin" [Willmott quotation]. If the locality data are incorrect, it is possible that the specimen is a *H. elevatus* x *H. pardalinus*.

Inírida is near the border with Venezuela, and I can't think of any reason that Schmidt-Mumm might have given this label information unless it really was from there. While this is the only *Heliconius* he obtained from this locality, he did also have Papilionidae specimens from there (Salazar 2002). Brower's suggested alternative parent,

Heliconius pardalinus, is known from Colombia, but the nearest locality is ~ 500 km to the South, and the species is not known from Venezuela at all (Brown & Fernández Yepez 1985). As such, a possible hybrid with *H. pardalinus* at Inírida is seemingly ruled out. The specimen lacks a characteristic yellow streak on the underside of the hindwing that distinguishes *H. elevatus* from *H. melpomene* with a very similar pattern. The lack of yellow streak is entirely expected because a hybrid may be heterozygous for such pattern characteristics, and the absence of yellow markings follows the expectation of red dominance over yellow for such yellow marks. In laboratory crosses of *H. pardalinus* x *H. elevatus* (Rosser *et al.* 2019), F1 hybrids similarly did not express the yellow underside hindwing streak. So I stand by our original assessment, as well as by the hesitancy that we expressed then about this unique specimen.

Schmidt-Mumm did purchase specimens from collectors, but they did not form the bulk of his collection. As far as I know no commercial butterfly breeding occurred in Colombia during the 1960s and 1970s when Schmidt-Mumm was primarily collecting. There were very few commercial tropical butterfly breeding operations worldwide until the 1990s. I believe that all of Schmidt-Mumm's specimens we cite are wild-caught, and not insectary-manufactured, and that most of the other collections from these regions in the 1980s and earlier are also legitimate. Yes, the commercial trade might possibly encourage production of hybrid rarities for the collectors' market. But there is strong evidence against the hypothesis that this happened in Colombia at the time Schmidt-Mumm was collecting, especially before the 1980s.

As Brower mentions, Mauricio Linares and Jesús Mavárez have also collected in another locality where putative hybrids frequently turned up, San Cristóbal, Táchira, Venezuela, which is just over the border with Colombia (it is near Cúcuta in northeastern Colombia). Dr. Mavárez provided me with a well-documented series of photos of the wings of these specimens, from among 103 *Heliconius cydno* and *H. melpomene* all captured together in the same locality (~7% of the population are colour-pattern hybrids!). Images of the hybrid specimens and a large sample of the parental forms are available in the supplementary material of Mallet *et al.* (2007; see file /mavarez/cristobaltab.html). Brower interprets these as follows:

> ... these specimens might represent not interspecific *H. cydno* x *H. melpomene* hybrids, but hybrids between *H. cydno cordula* and an unrecognized red-banded member of the *H. cydno* clade. In light of the genetic evidence [AFLP and microsatellite markers], and given the number of recently-discovered *H. cydno* cognates on the east side of the Andes, this hypothesis seems at least as parsimonious as an interspecific cross. Therefore, all of the Táchira specimens from the Mallet *et al*. (2007) database are interpreted as interracial, not interspecific hybrids, and given an identity score of zero.

Low resolution genetic data from a small sample of these individuals (Mavárez *et al.* 2006) indeed suggested late generation backcrosses to *H. cydno cordula*. However, Mavárez *et al.* apparently did not notice or discuss one *H. melpomene*-like hybrid, a likely backcross in the opposite direction (Mallet *et al.*, 2007, supplementary materials, file /mavarez/cxm007.html), that would also have been interesting to sequence, and which shows a clear brownish mark near the anal margin of the underside of the hindwing. This phenotype is found also in laboratory bred hybrids between *H. cydno* and *H. melpomene*, and is labeled *Brbr* in Fig. 1E of (Naisbit *et al.* 2003). For reference, this characteristic *Brbr* heterozygous *H. cydno* phenotype can be seen on the undersides of many of the other *H. cydno x melpomene* hybrids we reported in 2007: hybrid specimens 35, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 72, 86, 92. Nonetheless, Brower (2018a) assigns all of the hybrids from San Cristóbal and most of our databased hybrids very low identities and overall reliabilities; he believes they are all from *H. cydno* "cognates." Unfortunately, Mavárez *et al.* provided neither AFLP nor microsatellite data, but merely reported resultant STRUCTURE bar graph plots without identifying which bars belonged to which individuals. As accepted by Camilo Salazar, Mauricio Linares, Jesús Mavárez, Chris Jiggins, and myself, the aberrant individuals captured from this locality are almost certainly multigenerational backcross hybrids to *H. cydno* or in one case to *H. melpomene* for the following reasons:

(1) Brower's hypothesis requires postulating an unknown "red-banded member of the *cydno* clade" for the other parent, instead of the red-banded race of *H. melpomene* with which *H. cydno* actually is known to fly (see image collection in the supplement of Mallet *et al*., 2007). Brower's hypothesis seems highly unparsimonious.

(2) The nearest known red-banded member of the *tristero/timareta* group of species is *H. heurippa*, a hybrid species found at the closest around 250 km to the south, north of which it is replaced by *H. cydno cordula*. *H. cydno* has no red-banded race. Mauricio Linares has collected extensively along this Eastern slope of the Andes, and we

know of no region where *H. heurippa* or *H. timareta* and *H. cydno* overlap. Because *H. cydno cordula* is distributed along the northeastern flanks of the Andes in northern Colombia and into Venezuela, any contact with *H. heurippa* should occur near Yopal, Casanare. Mauricio Linares and I attempted to collect close to this region (on an earlier trip, Dr. Linares had been kidnapped in Yopal, and his vehicle was stolen), but we were unable to find any *H. cydno* or *H. heurippa* there.

(3) Very similar colour pattern phenotypes can be obtained readily in laboratory crosses between *H. melpomene* and *H. cydno* (Naisbit *et al.* 2003, Fig. 1).

(4) Two new well-studied subspecies of *H. timareta* (also *H. cydno* "cognates," as Brower calls them) are now known, one rayed and one red-banded from Southern Colombia and Northern Peru. In both cases there is clear molecular genetic evidence for relatively frequent hybrids with sympatric *H. melpomene* (Giraldo *et al.* 2008; Mérot *et al.* 2013). Indeed, both of these *H. timareta* subspecies appear to have acquired colour patterns via introgression from *H. melpomene* (*Heliconius* Genome Consortium 2012). Another *H. timareta* form, *H. timareta linaresi*, which is melanic with only a single yellow forewing band, is found in a restricted region of the Eastern Andes south of the range of *H. heurippa* near Villavicencio (Arias *et al.* 2017). It appears that *H. cydno* replaces *H. timareta sensu lato* (when we include *H. heurippa* within *H. timareta*) north of Yopal, and no further *H. timareta* are expected to occur as far north as San Cristóbal. To back up the case for recurrent hybridization and introgression between sympatric populations of *H. cydno* and *H. melpomene* in Panama, and between *H. timareta* and *H. melpomene* in Peru, we now have convincing genomic data that proves such introgression: around 40% of the genome has over perhaps many thousands of years been exchanged between these species in Panama, and at least 20% in Peru (Martin *et al.* 2013).

So yes, hybrids, especially between *H. cydno* and *H. melpomene* indeed seem rather frequent in certain sites in the Magdalena Valley, like Victoria and Otanche, and in San Cristóbal to the North. Hybrids between *H. melpomene* and *H. timareta* (another *H. cydno* "cognate") also appear to be rather common further south in Florencia, Colombia, and near Tarapoto, Peru. Elsewhere in Ecuador, Colombia, Venezuela, and Central America, *H. cydno* x *H. melpomene* hybrids are rare. I recently met and discussed the situation in Venezuela with a resident collector, Mauro Costa Cicognani. He knows Roberto and Renato Mattei, the sons of Otto Mattei, who was also a collector of several of the hybrids contested by Brower, and can confirm that they were never in the business of rearing butterflies; their specimens were all collected in the wild. They live in Puerto Ayacucho on the border with Colombia, and are now reputedly suffering terrible deprivations due to the political situation in Venezuela. Mauro Costa himself collected a *H. cydno barinasensis* x *H. melpomene melpomene* hybrid, again at San Isidro (Mina) 1500m, Barinas (cf. Mattei specimens, hybrid nos. 95-96), on 1 Jan 2006. Costa confirmed that this is the only interspecific *Heliconius* he has yet captured; these hybrids are indeed rare across most of Venezuela, as elsewhere. The fact that many hybrid specimens come with very good credentials, for example from Ernesto Schmidt-Mumm and Jesús Mavárez, or Jose Clavijo and the Mattei family in Venezuela, persuades me that most, if not all of the others, particularly those collected by José Urbina, must be treated as reliable as well. I have or had close ties to many of the collectors of these hybrids in the region, and I am convinced they were not in the business of manufacturing hybrids. So I stand by our original decisions for all those Colombian and Venezuelan hybrids.

Distant hybrids and DNA analysis of a wild caught hybrid between *H. ethilla* and *H. melpomene*

In order to support his skeptical stance on our evidence, Brower seems to go out of his way to dismiss the reliability of distant hybrids. This explains his preference for the idea that the Puerto Inírida hybrid mentioned above is between *H. pardalinus* and *H. elevatus* and not *H. hecale* and *H. elevatus*, because *H. hecale* is somewhat more distantly related to *H. elevatus*. It is why he postulates an undiscovered red-banded "*cydno* cognate" for one of the parents of the hybrid swarm at San Cristóbal, because it would be more closely related to *H. cydno cordula*. It is also why he pays special attention to even more distant hybrids, such as those between silvaniforms and *H. melpomene*, or between *H. erato* and *H. charithonia*.

Our boldest suggestion was about a putative *H. erato* x *H. charithonia* hybrid, no. 158. I first saw an image of this specimen in a book (de la Maza 1991), but have never seen the specimen itself. The only photos I had in 2007 were very low quality. I have recently received much better digital photos of this same specimen (Fig. 1). I still believe it is probably a hybrid. In contrast, Brower argues, to my astonishment, that it is an aberrant *H. charithonia*

and that it is unrelated to *H. erato*, even though Roberto de la Maza had originally listed it as a variety of *Heliconius erato petiverana*. The colours of the forewing band, both upper and underside, and their restriction to the exact region of the forewing where the red band of *H. erato* would be expressed in Mexican specimens, as well as the partial thin yellow hindwing bar, with an underside shadow that curls forward towards the margin just like the yellow bar in Mexican *H. erato*, surely argue for some *H. erato petiverana* ancestry to an expert on *Heliconius*! As we did, Brower recognizes the unusual elongated wing shape and undulate hindwing margin as *charithonia*-like, and this seems to be the main reason that he instead accepts the specimen as an aberrant *H. charithonia*. As we noted, this unique specimen represents the most phylogenetically distant hybrid we proposed, and there will always be some uncertainty without confirmation from molecular genetics. In the absence of such data, the unique pattern deviations in both fore- and hind-wings from the expected *H. erato* phenotype seem most parsimoniously explained by hybridization of *H. erato* with the abundant and sympatric *H. charithonia*, as explained in our original justification.

FIGURE1. A putative hybrid between *H. erato* and *H. charithonia* from Oaxaca, México
Photographs: By courtesy of Ivonne Garzón-Orduña, Curator, Colección Nacional de Insectos de México, UNAM.

Brower also dismisses all hybrid specimens we proposed between *H. melpomene* and silvaniforms, concluding that not a single such hybrid has a reliability score > 0.5. Again, the reason may be that Brower holds to a strong prior probability that such distant hybrids do not exist, rather than having major doubts with the visual evidence or known colour pattern genetics, or about the authenticity of the multiple specimens. By providing low scores on both elements of his reliability score, he is able to make these hybrids seem highly unreliable on his scale.

Occasionally in captivity, such hybrids occur in insectaries (Gilbert 2003). For example see Plate 1B in (Gilbert 1984), showing that silvaniform-*melpomene* hybrids are possible. Furthermore, once the species barrier has been breached, mating preferences involving F1 interspecific hybrids in *Heliconius* appear to be much less strong than those of their parents against mating with other pure forms (McMillan *et al.* 1997; Naisbit *et al.* 2001), as expected given approximately additive inheritance of both mating cues and mate preference. One enterprising amateur butterfly breeder, Jean-Pierre Vesco, obtained F1 hybrids in captivity between *H. hecale* and the silvaniform *H. atthis*, and then backcrossed these F1 hybrids to *H. hecale*, *H. atthis*, as well as to *H. melpomene* x *H. cydno* hybrids! Again we described this and showed photos of the hybrids, kindly provided by M. Vesco, in another online appendix (Mallet *et al.* 2007). Of course, insectaries are unnatural, but in these cases the hybrids were not forced, and given the insectary hybrids, the probability must also be high that such hybrids also occur occasionally in nature, where the parent species co-occur. Brower's skepticism about rare wild-caught hybrids must face this certain knowledge from insectaries of their probability.

Although I have not personally encountered any obvious silvaniform x *melpomene* hybrids in the field, my students and colleagues have. We sequenced DNA from mitochondrial and nuclear genes from a putative hybrid, specimen 06-921, and we therefore could prove that it is a hybrid, almost certainly a first generation hybrid between the silvaniform *H. ethilla* and *H. melpomene* (Dasmahapatra *et al.* 2007). To bolster support for his skepticism towards such distant hybrids, Brower sought to discredit our work with this specimen by criticizing almost every aspect of this publication. I therefore answer these criticisms in detail below.

First, Brower questions the specimen's authenticity. The collector was not reported! Well, this is true, and there's a story about that. Two of the authors of the paper, Jae-Woo Chung, a PhD student studying with me at University College London, and Armando Silva-Vasquez, a Peruvian colleague from the region, were both on this field trip to Rumiyacu, near Moyobamba, San Martín, Peru, at the latitude and longitude given in the original paper, and *both* claimed to have captured this hybrid. They could not agree with each other about who had actually captured the specimen. They were both on the field trip, and so there is no doubt the specimen was captured on this trip by one of them. It is almost impossible for two people to net the same specimen, so Kanchon Dasmahapatra, who carried out the DNA lab work, and I settled on the solution of including both as co-authors on the final paper.

Brower queries why the exact date was not shown "2006 (?)". The precise day and month was indeed an omission, but I can reveal from our lab database that this specimen was collected on 13 Dec 2006. The late capture date in the year 2006 explains why this specimen was not included in the main database of Mallet *et al*. (2007), since that paper had by then already been finalized and submitted to BMC Evolutionary Biology.

Next Brower casts doubt on our handling of the molecular data:

> ... Genbank accession numbers were not reported in Dasmahapatra *et al*. (2007).

This is not true. We supplied the Genbank IDs (AM709679-AM709838) in the supplementary material to the paper. However, Brower seems to have found the data anyway.

The mitochondrial DNA sequence for 06-921 showed clearly that it had an *Heliconius ethilla* mother, which Brower accepts. The nuclear data, of course, are key to providing cast-iron evidence for hybrid status, and for the parentage of this aberrant specimen. The most likely species we knew from this region that could generate the specimen's *Heliconius* "hippola"-like pattern by hybridizing with *H. ethilla* was *H. melpomene amaryllis*, a common form in the Río Mayo valley near Moyobama and Tarapoto. (*Heliconius hippola* Hewitson 1867 is today generally recognized to be an *H. ethilla* x *H. melpomene* hybrid. It was described and illustrated from a specimen from Colombia; the type at the BMNH is listed in Mallet *et al*. (2007) as hybrid specimen no. 17; needless to say, Brower assigns a very tiny overall reliability score of 0.04 to this historic specimen).

Unfortunately, Sanger sequencing is not single-molecule sequencing, so with approximately equal titres of paternal and maternal haplotypes, each heterozygous site would lead to two overlapping chromatogram peaks of

approximately the same height. The chromatograms were of very high quality with the ABI 3730xl machine we used, so we could readily check for heterozygous sites (Fig. 2). As well as the hybrid, we also sequenced possible pure parents for comparison for several nuclear loci. Brower critiques the approach as follows:

> ... several of these genes are known to exhibit dramatic heterozygosity of intron sequence and length within "pure" (i. e., not hybrid) individuals ..., yet Dasmahapatra *et al*. (2007) used single sequences of *H. ethilla* and *H. melpomene* in their Neighbor-Joining analyses, apparently assuming that every other specimen except 06-921 was homozygous.

No assumption of homozygosity was in fact made, as Brower could have checked if he had examined our sequence data more carefully. All of the loci have heterozygous base calls (we used two-fold IUPAC ambiguity codes to call heterozygotes; e.g. Fig. 3) in some of our pure species sequences, and most loci have them in many sequences. Our sequences of mitochondrial and nuclear loci were BLASTed against all sequences at that time on Genbank (See Table S2 in the supplementary material of Dasmahapatra *et al.* 2007).

Most of the nuclear sequences were across introns, with primers located in conserved coding sequence. The procedure involved PCR amplification of the sequence of interest, followed by Sanger sequencing. As Brower mentions, with sequences heterozygous for indels, Sanger sequencing can lead to highly scrambled sequence information, because the multi-molecules being sequenced no longer match at aligned homologous positions after the beginning of any heterozygous indel. Because a hybrid is likely to be heterozygous, and as we show in this case, indeed is highly heterozygous for any fixed differences in indels as well as for substitutions, this meant that our intron data for the hybrid could be highly scrambled.

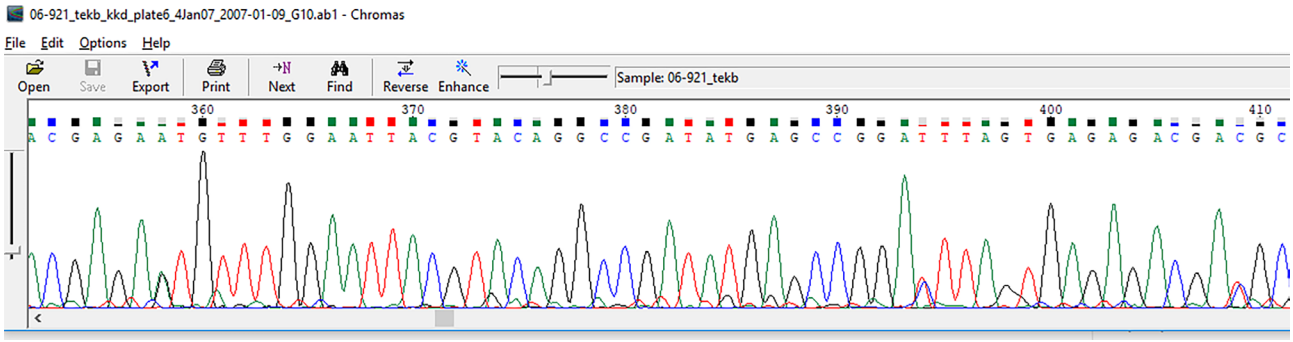

FIGURE2. Chromatogram of a heterozygous DNA sequence from our ABI 3730 xl sequencer
This partial chromatogram is from a *Tektin* exon in our study of hybrid specimen 06-291. Note the very clean chromatogram peaks for each site. Three sites are shown that are clearly two-fold ambiguous (i.e. heterozygous): 358, 394, and 409. These were scored as R (i.e. A-G heterozygote), Y (i.e. C-T heterozygote), and Y, respectively. This RYY corresponds to the central section of the 06-291 hybrid sequence shown in Fig. 3.

At this time a classic method to deal with such scrambled heterozygous information was to clone the gene region of interest. By growing up multiple clones of the region from a single heterozygous individual, one hoped to be able to sequence both alleles. However, cloning PCR products had its own problems. DNA polymerase used in PCR generates errors. Sanger sequencing of PCR products, which are mixtures of the family of correctly copied and erroneous sequences normally works well, because the PCR errors are not common and are distributed along the sequence, so that the signal-to-noise ratio is still good. Although PCR, cloning, and then sequencing will be essentially equivalent to single molecule sequencing, it will also fix any errors introduced in the first PCR stage. This was the tactic we used earlier to deal with nuclear intron sequences, and we discovered a number of such cloned PCR errors, including clear *in vitro* recombinants between alleles (Beltrán *et al.* 2002; Kronforst *et al.* 2006). In practice one must therefore sequence multiple clones from the same individual, and decide on a consensus sequence for each allele based on the assumption that errors will be scattered and rare per site (Bull *et al.* 2006, p. 13). So, while cloning allowed one to "phase" heterozygous sequences with Sanger sequencing, it was error-prone, very laborious, and around 10x as expensive as sequencing the PCR products directly.

However, we found an alternative method for deconvoluting the two alleles in heterozygotes. Brower complains about this as follows:

> Teasing apart a chromatogram with heterozyous sites into two separate alleles is not an easy feat, but other than some description of "deconvolution" of sequences of variable length, Dasmahapatra *et al*. (2007) did not describe how they determined the sequences of alternate alleles, other than by comparing the heterozygous sites to the sequences of the two putative parental species.

Teasing apart a chromatogram with heterozygous sites is indeed difficult. If a single heterozygous pair of sequences is collinear without indels (Fig. 2), there is no way of phasing the heterozygous sites to obtain the original maternal and paternal alleles without cloning, but an alternative is to simply report the heterozygous bases in the Genbank record, as we did for our pure species sequences, and for the hybrid, for the coding sequence for *Tektin* (see below). However, after getting scrambled reads of heterozygous introns, we suddenly realized that indels might allow unscrambling, or "deconvolution" as we called it.

If there is a heterozygous indel in a diploid individual, double chromatogram peaks would begin to accumulate along the Sanger sequence within and after the indel much more frequently, as the maternal and paternal allele sequences went out of alignment. This suggests that solving a Sudoku-like puzzle could be used to phase the alleles. If there is one indel, one can sequence a PCR product from both ends, and assume that the indel starts where the sequence chromatogram first becomes scrambled from each end. Then the puzzle is to move the scrambled heterozygous sequence with the heterozygous bases along one base at a time and to find the indel length that produces the maximum number of matches with one base of each heterozygote call from where the clean sequence ended. I personally found this was easiest to do by cutting out paper copies of the mixed-signal chromatograms from the beginning of the scrambled part, and then moving the cut-out along a sequence generated by the complement of the reverse sequence, one base at a time until the sequences matched maximally.

This led to a remarkable finding: whereas we could not phase heterozygous sequences without indels for a single individual as in Fig. 2, if there was an indel in one of the alleles, we could phase the sequence almost perfectly (with any alignment procedure for indels, there will always be potential ambiguities near the indel break-points depending on the exact sequence itself). An indel imperfection in the heterozygous sequence yields deconvolution, whereas a sequence without such an imperfection cannot!

We were excited by this result, and at first believed we had made a new discovery. However, we failed in an attempt to automate the procedure, and we soon discovered that another laboratory had already discovered this same deconvolution method (Flot *et al.* 2006). Therefore, in our paper (Dasmahapatra *et al.* 2007), we mentioned what we did, but, as is normal in scientific publication, referred for the method to Flot *et al*. (2006), rather than describing in detail the method again. We then reported both de-convoluted sequences from the hybrid separately on Genbank.

Unfortunately, with more than one heterozygous indel, a non-automated approach to deconvolution becomes increasingly difficult. However, here a different approach can be adopted. If homozygous sequences are available from the same species, indel differences among the homozygotes can be used to phase sequences heterozygous for indels. In essence, this is related to the population genetics approach used in many programs to phase heterozygotes (Choi *et al.* 2018). Here, linkage disequilibrium among loci from population data is being used in a statistical framework to determine likely haplotypes from heterozygous individuals. If the heterozygote is a hybrid between two species with multiple fixed indel differences, then the homozygous pure species sequences can similarly be used to help unscramble or phase the hybrid. The hypothesis test is to find whether the scrambled sequence can be explained as a heterozygote between different pure species sequences of putative parents with different indels fixed. If it can be explained this way, then this is very strong evidence that the scrambled sequence is actually a heterozygote produced due to hybridization between different species. This is certainly another procedure we adopted for *H. ethilla* x *H. melpomene* 06-921.

Returning to Brower's critique:

> Needless to say, calling the bases so that they match one or the other parental sequence is hardly an independent corroboration of the allelic similarity of the "hybrid" to the parents.

Brower suggests that we "call[ed] the bases so that they match[ed] one or the other parental sequence". Instead, we tested the hypothesis that scrambled (i.e. heterozygous) regions could be explained as a result of a heterozygote in Sanger sequence data between DNA haplotypes from two putative parental species with different indels. We

found that they could. Isn't this an example of a recognized major way of doing of science? We tested whether the data fits better with one hypothetical model (in this case hybridization) than an alternative (in this case a lack of hybridization).

Brower complains that not all the data used in Dasmahapatra *et al*. (2007) were placed on Genbank, as follows, and uses this opportunity to refer to another of his own critical papers of my colleagues and myself, where he had previously also made clear his displeasure at any lack of provision of data:

> .... some *Mpi* sequences included in Dasmahapatra *et al*. (2007) fig. 2 are not present in GenBank. These problems cast doubt upon the value of these sequences as evidence for interspecific hybridization (cf. Brower 2011).

However, in contrast to Brower's assertion, I can confirm that all of the *Mpi* sequences are on Genbank, but that two of them were mislabeled as to specimen ID; this is probably why Brower did not find them in the Genbank records adjacent to the other *Mpi* sequences from these specimens. AM709817 and AM709818 sequences from *H. melpomene aglaope* should have been labeled 04-286 and 04-288 as opposed to 02-286 and 02-288 as listed in Genbank.

Brower again:

> Most *Heliconius invected* sequences in Genbank are over 400 bp long, and Dasmahapatra *et al*. offered no explanation why these sequences are so truncated [52-53 bp long].

Sometimes, the indels are so many, and so complex, including polymorphisms for multiple indels within species, that the sudoku-like puzzle for deconvolution becomes almost impossible. This was the case for *invected*, and was the reason we could not reconstruct pure sequences for more than a fragment of the entire intronic region of this hybrid. Brower's conclusion for *invected*:

> In any event, these tiny sequences do not provide much evidence for any pattern at all.

In spite of the shortness of the sequence that we could deconvolute, it is clear that the two copies in 06-921 at the *invected* locus as reported by us do strongly support the contention that these sequences were from a hybrid between *H. melpomene* and *H. ethilla* from this region of Peru, based on the parental sequences we provided, and so there was every reason to include these truncated sequences as evidence for hybrid status of this specimen.

> Note also that the last 9 bases of these two sequences are not parsimoniously viewed as homologous sites when aligned with longer *invected* sequences from other *Heliconius*.

Yes, the final 9 bases for these two truncated *invected* sequences seem most likely to be non-homologous due to two small additional deletions in the *H. ethilla* haplotype over and above the single base *H. ethilla* deletion shown by Brower (easily seen, for example, by aligning with the DQ448475 *H. hecale* sequence). Indels inferred are shown as bars on the trees shown in Fig. 2 of Dasmahapatra *et al*. (2007). Indels provide particularly good parsimony support for phylogeny, because compared with, say, a transition substitution, there is generally a very low likelihood of exactly the same indel arising twice in a closely related group of species. Unfortunately for Brower's argument, the lack of homology between the final 9 base pairs of our sequences between species, and within the hybrid, thus adds rather strong support for, rather than detracting from our argument for hybrid status.

We also sequenced one 740 bp coding region in the *Tektin* gene in the hybrid and parental species (Figs. 2,3). We had previously found this locus to be useful in phylogenetic studies as it coded for a sometimes relatively rapidly evolving structural protein (Whinnett *et al.* 2005). Because of a lack of indels, we were unable to use indel-based deconvolution to separate sequences, and so our putative hybrid sequence for this locus was reported on Genbank via two-fold ambiguity codes. Brower's complaint here is:

There is only one *Tektin* sequence in Genbank for 06-921 (AM709690), and Dasmahapatra *et al*. (2007) apparently sorted ambiguous chromatogram peaks from this sequence to match either an *H. melpomene* or an *H. ethilla* sequence. As noted above, such a procedure begs the question of the specimen's identity, and does not constitute evidence.

Brower's statement that the sequence "... does not constitute evidence" seems most odd. I show below that the sequence data provide remarkably clear evidence.

In Fig. 3, an alignment of all variable sites in the *Tektin* sequence data used in Dasmahapatra *et al.* (2007) is shown, together with comparison sequences from other species in newer whole-genome data. *Heliconius timareta thelxinoe* (Mérot *et al.* 2013), which inherited homologous colour pattern genetics with *H. melpomene amaryllis*, almost certainly via adaptive introgression (Mérot *et al.* 2013; *Heliconius* Genome Consortium 2012), might also have been a parent, as it could have led to similar colour pattern distortions in the putative hybrid 06-921. As we can see, the assembled *H. timareta* genome sequence had a *Tektin* sequence identical to the consensus sequence for *H. melpomene* from the region. (However, from fixed differences at other loci, for example from *Tpi*, we can rule out *H. t. thelxinoe* as the male parent).

```
Lepbase H. timareta helico3 scaffold 13388      ACTAATAGCAATTGGACATGA

AM709679 H. melpomene amaryllis 02-1882         ACTAATAGCAATTGRMCATGA
AM709680 H. melpomene amaryllis 02-1850         ACTAATAGCAATTGGACATGA
AM709681 H. melpomene amaryllis 02-944          ACCAATAGCGATTGGACAYAA
AM709682 H. melpomene aglaope 02-2060           ACYAATAGCRATTGGACATGA
AM709683 H. melpomene aglaope 02-1894           ACTAATAGCAATTGRCCATGA
AM709684 H. melpomene aglaope 02-366            ACTAATAGCAATTGGACATGA
AM709685 H. melpomene aglaope 02-288            MCYWATAGTRATTGGACATGA
AM709686 H. melpomene aglaope 02-286            ACTAATAGCAATTGGACRTGA

AM709690 H. ethilla x H.melpomene 06-921        AYTARYRKYARYYRGAYATGR

AM709687 H. ethilla aerotome 02-3               AYTAGCGTTAGCCRGATATGG
AM709688 H. ethilla aerotome 02-975             AYTAGCGTTAGCCRGATATGG
AM709689 H. ethilla aerotome 02-1483            ACTAGCGTTAGCCGGATATGG

Lepbase H. pardalinus helico3 scaffold 6955     ACTAGCATTAGCCGGACATGG
```

FIGURE3. Variable sites within the 740 bp *Tektin* sequence data.

For the Genbank sequences from *H. melpomene*, *H. ethilla*, and the putative hybrid specimen, all variant sites are shown in order along the 740 bp *Tektin* partial sequence, with uninformative fixed intervening bases omitted. Variants and two-fold ambiguous (i.e. heterozygous) sites are shown in bold. The following two-fold IUPAC ambiguity codes were found in hybrid 06-921: Y = C/T, R = A/G, K = G/T. For comparison, also shown are aligned bases obtained by BLAST for the haploid whole genome assemblies in Lepbase 4 from two related species *H. timareta*, and *H. pardalinus* (Challis *et al.* 2016).

As seen in Fig. 3, specimen 06-921 has three times as many heterozygous sites at *Tektin* as any other specimen from either species; its overall heterozygosity at this locus is 0.81% compared to average expected heterozygosities in *H. melpomene Tektin* of 0.16% and for *H. ethilla* of 0.09%. There is nine times as much *Tektin* base heterozygosity in this specimen as the average in *H. ethilla*, from which species Brower argues specimen 06-921 and other "hippola"-like forms might be variants. As reported in Dasmahapatra *et al*. (2007), for all 9 sites that show a fixed difference between *H. melpomene* and *H. ethilla*, specimen 06-921 yielded exactly the double chromatogram peaks expected if heterozygous for those sites (see Fig. 2, which shows the central RYY portion of the variant calls in Fig. 3), as they should if this was indeed a hybrid between these two species. If these were instead just a result of scattered variants among four *H. ethilla* individuals sequenced, which seems to be Brower's favoured alternative, the probability that they all accumulated as random heterozygotes in the same individual is something like $P = (1/4)^9 = 0.0000038$. Of course, this ignores possible linkage disequilibria within species, but in this case it is pretty clear that the strong linkage disequilibrium observed is due to species differences, in other words, that the specimen is a hybrid.

We sequenced five nuclear loci as part of this project for both the putative hybrid and parental sequences. Of these, four loci show clearly the expected multi-site heterozygous pattern, exactly as expected if this specimen was indeed an F1 hybrid between the two species reported. The fifth locus, *rpl5* is consistent with having a heterozygous sequence between the two species, but, as noted in our original write-up, does not prove it in the same way as the other four, due to lack of phylogenetic resolution between these two species at this locus. If these same four heterozygous sequences are to be explained by polymorphism within *H. ethilla*, we are required to believe, on the other hand, a very large set of extraordinarily special postulates about variable sites, all of which must somehow converge in this single specimen, a specimen that also just happens to be morphologically highly aberrant.

In his summary of evidence for this specimen, Brower concludes:

> The molecular evidence thus leaves room for doubt about the plausibility of this specimen's hybrid origin.

Brower's doubts are apparently sufficient to reduce his own identity score to 0.75, and his doubts about the provenance of the specimen allow him to reduce his authenticity score to 0.9 due to what he calls "vague details" of its collection. When multiplied together, the two yield an overall reliability of 68%, which is below Brower's stricter cut-off reliability threshold of 75% (as reported in Brower's Table 1). Therefore, together with his rejection of the many other specimens, Brower can maintain that there are *no* reliable (i.e. > 75% reliability) wild-caught hybrids between any silvaniform and *H. melpomene*. In contrast, we find it very hard to doubt from the data reported that specimen 06-921 is a wild hybrid. The clarity of our finding, coupled with insectary evidence of hybridization makes one prone to believe that the majority of other wild-caught specimens reported by Mallet *et al*. (2007) and based on morphological evidence alone are wild-caught hybrids as well.

I felt it necessary to lay out our reasoning in the analysis of this single specimen in such excruciating detail because Brower questioned every single aspect of the data and our interpretations. There might be a chance that Brower's paper may convince doubtful readers that my colleagues and I are attempting to persuade other scientists that *Heliconius* species hybridize much more than they really do, and that we backed this up by dubious reasoning about a possibly falsified molecular dataset for this specimen. In my view, our brief report on molecular genetic data (Dasmahapatra *et al.* 2007) was already quite sufficient to establish that the specimen 06-921 was captured in the field by our team and that it is a hybrid between *H. ethilla* and *H. melpomene*.

Discussion and conclusion

Twelve years ago, in our 2007 papers, my colleagues and I documented overwhelming evidence for occasional hybridization among closely related species in the wild. We demonstrated by means of morphological criteria the evidence for hybridization in the wild in many heliconiine specimens from museums and private collections from around the world, from many different localities, with specimens collected by many collectors (Mallet *et al.* 2007). We also showed using the best available molecular technology at that time that an unusual wild-caught specimen collected in the field by our own group was explicable only as a hybrid between two somewhat distantly related non-sister species (Dasmahapatra *et al.* 2007). This well-documented specimen provides hard molecular genetic evidence for the existence of wild hybrids as distant as between these two non-sister species, *Heliconius ethilla* and *H. melpomene*.

Even supposing we do accept Brower's conclusions at the strictest level that even he seems to believe is necessary (overall Brower score reliability > 75%, his Fig. 181), then we still have 12 species hybridizing. Given that today there are 48 known *Heliconius* species (Lamas & Jiggins 2017) his conclusion would presumably be that ~25% of species currently hybridize in the genus, in other words, roughly what we had estimated in 1998 (Mallet *et al.* 1998b).

Perhaps because of this, in the end Brower's conclusions against the pervasive nature of hybridization among species in this genus seem rather mild. Hybridization – it does occur in the wild, yes, but according to Brower it's a little less frequent on a per species basis than we had argued, 25% instead of 33% of species indulge in it using his draconian > 0.75 reliability score. He concludes:

> Reconsidering this source of raw evidence for interspecific hybridization suggests that the phenomenon occurs much less frequently than Mallet *et al*. (2007) proposed.

As already mentioned, at the same time, Brower still seems to use these same conclusions to deny strenuously that any hybrids between species are possible. Although Brower accepts that hybridization occurs, this doesn't stop him from making comments like this:

> Thus, hybridization among *Heliconius* species persists as a hypothetical phenomenon in a cryptobiological sweet spot, occurring too infrequently to detect in experiments, and, as shown here, supported by relatively scant evidence from collections and the field, much of which is, to say the least, subject to alternative interpretation.

Or this:

> As the empirical foundations of interspecific hybridization between *Heliconius* species erode, the credibility of widespread introgression diminishes, and traditional explanations for Müllerian mimicry among *Heliconius* butterflies, such as adaptive convergence, must be reconsidered.

In the first paper (Mallet *et al.* 2007), we examined aberrant wild-caught specimens of heliconiine butterflies in an effort to estimate the probability of hybridization among species. In contrast, Brower's strong personal prior probability beliefs against hybridization lead him to take the stance that any doubt in his mind about a particular aberrant specimen invalidates even the most striking evidence for hybridity. On a per-individual basis, hybridization certainly is rare, as we documented. But that doesn't mean that it never occurs. Using his scoring procedure Brower is therefore able to cast doubt on many of the hybrid specimens, even though he agrees that hybrids do occur in the wild among closely related species like *Heliconius cydno* and *H. melpomene*.

We never claimed that "traditional explanations for Müllerian mimicry among *Heliconius* butterflies, such as adaptive convergence" are invalidated by our findings of introgression. Although genomic data shows that colour pattern-determining alleles have been transferred among closely related species, and appear to have aided Müllerian mimicry by introgression of whole adaptive cassettes (*Heliconius* Genome Consortium 2012; Wallbank *et al.* 2016; Jay *et al.* 2018), genomic evidence also suggests that mimicry between the more distant species, such as *H. melpomene* and *H. erato*, occurred purely as a result of adaptive convergence (Van Belleghem *et al.* 2017).

Why does Brower not believe our work on the hybrid question? It perhaps seems to him that my colleagues and I are unfairly promoting the evolutionary importance of hybridization. To support his skeptical narrative, Brower and assistant reanalyzed my colleagues' laboratory crossing data between *Heliconius* species, in terms both of assortative mating and hybrid sterility. Brower concludes that the species studied are "completely reproductively isolated," based on an overall measure of "reproductive isolation"

> ... indeed, the *Heliconius* species in question are, from a quantitative perspective, completely reproductively isolated (Garzón-Orduña & Brower 2018; Brower 2018b).

Therefore, he seems to argue, hybridization simply cannot happen. This is tantamount to believing the fallacy that if something is rare, it doesn't exist. And this is very odd, because lab-reared hybrids were used to make these estimates of reproductive isolation, proving that they can exist. Logic like this might lead to other similar beliefs, such as that because mutation is rare it can't contribute to variation and evolution in natural populations. The problem is that the measure of "reproductive isolation" used in the reanalysis by Garzón-Orduña & Brower of interspecific crosses in the lab is not a very useful way of finding out whether rare hybrids do occur and lead to introgression in nature.

I stand by our 2007 estimates of the fractions of species of heliconiines and *Heliconius* that are known to hybridize in the wild, around 26-29%, and 33-35%, respectively (Mallet *et al.* 2007). I also stand by our clear genetic demonstration that specimen 06-921 is a wild-caught hybrid between *H. melpomene* and *H. ethilla* (Dasmahapatra *et al.* 2007). Given that not all localities have been well-collected in the remoter parts of the neotropics, I do not doubt that the actual fraction of species of Heliconiini that hybridize has been somewhat underestimated, rather than overestimated as Brower seems to believe.

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