Disruption of the odorant coreceptor Orco impairs foraging and host finding behaviors in the New World screwworm fly

Daniel F. Paulo1, Ana C. M. Junqueira2, Alex P. Arp3, André S. Vieira4, Jorge Ceballos5, Steven R. Skoda6, Adalberto A. Pérez-de-León7, Agustin Sagel6, William O. McMillan8, Maxwell J. Scott9, Carolina Concha8,10 & Ana M. L. Azeredo-Espin1,10

The evolution of obligate ectoparasitism in blowflies (Diptera: Calliphoridae) has intrigued scientists for over a century, and surprisingly, the genetics underlying this lifestyle remain largely unknown. Blowflies use odors to locate food and oviposition sites; therefore, olfaction might have played a central role in niche specialization within the group. In insects, the coreceptor Orco is a required partner for all odorant receptors (ORs), a major gene family involved in olfactory-evoked behaviors. Hence, we characterized the Orco gene in the New World screwworm, Cochliomyia hominivorax, a blowfly that is an obligate ectoparasite of warm-blooded animals. In contrast, most of the closely related blowflies are scavengers that lay their eggs on dead animals. We show that the screwworm Orco orthologue (ChomOrco) is highly conserved within Diptera, showing signals of strong purifying selection. Expression of ChomOrco is broadly detectable in chemosensory appendages, and is related to morphological, developmental, and behavioral aspects of the screwworm biology. We used CRISPR/Cas9 to disrupt ChomOrco and evaluate the consequences of losing the OR function on screwworm behavior. In two-choice assays, Orco mutants displayed an impaired response to floral-like and animal host-associated odors, suggesting that OR-mediated olfaction is involved in foraging and host-seeking behaviors in C. hominivorax. These results broaden our understanding of the chemoreception basis of niche occupancy by blowflies.

Identifying the genomic changes underlying adaptation in living organisms is a fundamental goal of modern evolutionary biology. In the context of insect evolution, the emergence of parasitic lineages offers a unique opportunity to investigate the genetic basis of adaptive traits driving species to occupy novel ecological niches. As noted by Grimaldi and Engel (2005), "Calyptrate flies have redefined the 'art' of vertebrate parasitism, particularly the Oestroidea [...]," a superfamily that includes blowflies, botflies, fleshflies and relatives. Within Oestroidea, the family Calliphoridae is of particular interest due to its synanthropic habits. Commonly known as blowflies, the members of this family are frequently found foraging on plant inflorescence, where they feed on nectar, as well as breeding on decaying organic matter and carcasses. Therefore, blowflies have important roles in nature as pollinators and as recyclers of organic waste. In addition, they also have a remarkable importance in forensics, veterinary, and medical sciences. Although better known for these necro-saprophagous flies, the group spans...
an even greater diversity of life-history strategies, including many forms of parasitism (examples are given by\(^7\)). In particular, the evolution of obligate ectoparasitism is one of the most outstanding events within Calliphoridae, which has intrigued entomologists and evolutionary biologists over decades\(^\text{21,21}\). Estimations of the divergence timescale support that this lifestyle arose recently and independently at least twice after the explosive radiation of blowflies about 22 million years (Ma) ago, having a free-living scavenger ancestor\(^\text{10,12,13}\). The diversification of grazing mammals during this period is thought to have created a favorable landscape for ephemeral carrion resources. This harsh condition may have initially favored opportunistic blowflies attracted by decaying flesh in the surface of animal's wounds to oviposit, ultimately leading to the evolution of obligate ectoparasite lineages\(^\text{14}\). However, while these studies have addressed the likely origins of obligate ectoparasitism in blowflies, there have been relatively few attempts at understanding the genetic basis underlying this lifestyle.

Here, we hypothesized that olfactory chemoreception may have played a critical role in the adaptive transition from a necro-saprophagous to an obligate ectoparasitic habit in blowflies. Olfaction is a core chemosensory process in sensory perception, and divergences in olfaction-related genes are known to contribute to premating isolation, speciation and niche adaptation in insects\(^\text{35,38}\). We adopted the New World screwworm, Cochliomyia hominivorax (Coquerel 1858), as our research model. The screwworm is the sole obligate ectoparasite among the Cochliomyia genus, which includes four endemic species to the Americas, in addition to nearly all of the closely related blowflies, which are primarily carrion feeders\(^\text{21,17}\). Adult screwworms feed on flower nectar while their larvae feed on the live tissues of animals. Gravid female screwworms rely on odors emitted from wounded warm-blooded vertebrates to find suitable hosts for oviposition\(^\text{33,38}\), and lay their eggs on the dried margins of wounds and bodily orifices of their selected animal hosts. After hatching, the larvae infest and consume the animal living tissues to complete their development. These traumatic infestations are known as myiasis, which can lead to death if untreated\(^\text{5}\). The devastating effects of this decades-long eradication campaign, using the sterile insect technique (SIT), that successfully eliminated screwworms from North and Central America\(^\text{20}\). At present, millions of radiated-sterile screwworms are released daily along the Panama–Colombia border. This barrier zone prevents and counter-strike C. hominivorax outbreaks, as recently seen in the United States\(^\text{21}\), from regions where the species remains endemic. Meanwhile, screwworms remain a serious problem for animal health in South America\(^\text{3,22}\). Because of its well-established phylogenetic status, its consequences as a destructive invasive pest, and the increasing availability of genomic resources\(^\text{23,24}\), C. hominivorax represents a promising model to address the genetic basis of niche occupancy in blowflies.

In insects, the sense of odors in complex environments is mediated by several chemosensory genes expressed in porous sensilla attached to olfactory organs, including the antennae and maxillary palps\(^\text{25}\). Inside the sensilla, odors are solubilized and shuttled through the inner lymph to receptor sites present in the olfactory sensory neurons (OSNs). Two distinct receptor families, named ionotropic receptor (IR) and odorant receptor (OR), are responsible for recognizing the intercepted signals, resulting in the activation of OSNs and a cascade of neural events leading to a multitude of behavioral responses\(^\text{16,25–28}\). Although the OR and IR gene families include a large number of members (for instance, 60 and 66 genes in D. melanogaster, respectively\(^\text{29}\)) the proper number of all divergent ORs is dependent on a common odorant receptor coreceptor, named Orco\(^\text{27,30,31}\). A failure to encode Orco results in abnormal behaviors driven by OR-mediated olfaction, while maintaining other chemosensory pathways intact\(^\text{32–37}\). In this context, an Orco knockout strain of C. hominivorax would provide a simple model to rapidly differentiate olfactory behaviors mediated by the OR and IR families in this species. In this study, we isolated the Orco orthologue of C. hominivorax (named ChomOrco), characterized its sequence in a phylogenetic context, and assessed its developmental and tissue expression patterns. We next expanded our previous CRISPR/Cas9 genome editing protocols\(^\text{25}\) to develop a germline Orco null strain, and evaluated the contribution of OR-mediated olfaction in foraging and host-seeking behaviors in C. hominivorax. The data presented here provides new functional evidence on the chemoreception basis of ecological specialization in the screwworm fly.

**Results**

The screwworm Orco orthologue is highly conserved within Diptera. Classic-RACE was used to isolate the full-length Orco transcript sequence of C. hominivorax, which consists of 1437 base pairs (bp) encoding a 478 amino-acid (aa) peptide sequence. The ChomOrco transcript shares 73 and 92% of nucleotide identity with Orco sequences from D. melanogaster and the closely related Oriental latrine blowfly, Chrysomya megacephala, respectively. In addition, the ChomOrco coding sequence shows an extremely high aa identity with all dipterans investigated in this study (mean ± SD: 92 ± 5.5%; Supplementary Table S1). Based on its transcript sequence, we next used a combination of bioinformatics genome-wide analysis and long-range PCR sequencing to isolate the complete 12,870 bp genomic region corresponding to the Orco gene in screwworm. Genomic organization of ChomOrco is characterized by the presence of seven exons, highly conserved among dipterans, separated by six introns (Fig. 1A and Supplementary Fig. S1). Differently from D. melanogaster (DmelOrco), the exon 2 of screwworm Orco is subdivided into two parts (referenced here as E2a and E2b) separated by a 74 bp intronic region (named 11b). We further investigated the presence of intron 11b in other species and found that this region is not unique to the C. hominivorax genome, but rather has an ancient origin of at least ca. 50 Ma within the Sarcophagidae family, presumably being shared by all Calyptratae flies (Supplementary Fig. S1). Membrane protein topology predictions indicated that ChomOrco contains seven transmembrane domains (TM), an inverted terminal membrane topology (N\text{term}-C\text{term}) and a conserved tyrosine residue at TM7 (Fig. 1B), all signatures of this atypical OR coreceptor\(^\text{27,38}\). Most conserved residues were found at TM6 and TM7, which is thought to be a region where ORs partially interact with Orco. As evidenced in other calyptrates\(^\text{28}\), ChomOrco is eight amino acids (\text{PSPNGGGGNGL}) shorter than Drosophila at the intracellular loop 2 (IC2), which connects the
TM4 and TM5, a region believed to be important for intracellular transport\textsuperscript{27}. The long IC2, in comparison with the conventional ORs, is another Orco feature\textsuperscript{31,40} and this region appears to be a common place for Orco length variations in dipteran species (Supplementary Fig. S1). Gene tree inferences recovered ChomOrco in the Calliphoridae clade sharing a common node with Orco sequences of Muscidae species. The clade composed of Calliphoridae + Muscidae forms a sister-clade with Drosophilidae or Tephritidae families (Fig. 1C), consistently reflecting the phylogenetic relationships among the Schizophora clade\textsuperscript{10}. We investigated the branch leading to \textit{C. hominivorax} species for site-specific signatures of episodic diversification, as positive selection pressures are likely to affect only a few sites within a specific lineage. Results revealed that the selective pressures in the screwworm lineage do not differ from the background tree, presenting signals of a strong purifying regime (ω\textsubscript{K0} = 0.025) in the majority of the corresponding aa sites (97.3%), while remaining sites showed evidence of relaxed constraint (ω\textsubscript{K1} ∼ 1; Fig. 1B,C). Only a single site (211threonine, located inside TM4) exhibited some signal of positive selection in the \textit{Cochliomyia} clade (ω\textsubscript{frg} ≥ 1, while ω\textsubscript{bkg} < 1), although it was not statistically significant (BEB score: 0.92). Thus, it’s likely that this site is rather experiencing a relaxation of selective constraints in this clade. The same evolutionary signatures were obtained when testing the branch leading to \textit{Drosophila suzukii} lineage (DsuzOrco; Fig. 1C), illustrating that the Orco sequence conservation reflects its indispensable role in olfaction across taxa.

Figure 1. Screwworm Orco orthologue is highly conserved within diptera. (A) Comparison between the Orco genomic organization in \textit{D. melanogaster} (DmelOrco) and \textit{C. hominivorax} (ChomOrco). Exons are represented by numbered black boxes (E1-E6) and introns as connecting lines (I1-I5). The seven transmembrane domains (TM1-TM7), conserved tyrosine residue at TM7 (Y), and nucleotide length variation at E3 (gray strip) are also represented (dipteran’s Orco sequence alignment is shown in Supplementary Fig. S1). (B) Predicted protein topology of ChomOrco displaying significant characterized sites by Bayes Empirical Bayes (BEB). (C) Maximum Likelihood (ML) reconstruction of relationships between dipteran Orco sequences (species and accessions used can be found in Supplementary Table S3). Bootstrap support values, estimated from 500 non-parametric replications, are shown at their respective nodes. Normalized non-synonymous (d\textsubscript{N}) to synonymous (d\textsubscript{S}) substitution rates (ω) were estimated to test the branches leading to \textit{C. hominivorax} (in green) and \textit{D. suzukii} (in blue) species for events of episodic diversification. The number of sites estimated to be evolving under purifying (K0), relaxed (K1), and positive (K2a or K2b) selection is shown for these lineages (foreground branch; ω\textsubscript{frg}) in relation to the rest of the tree (background branch; ω\textsubscript{bkg}).
Expression of *Orco* is conserved among blowflies and broadly detected in chemosensory-related tissues of *C. hominivorax* adults. (A) Relative expression of ChomOrco during the screwworm development by qPCR. Measurements are given by the quantification of ChomOrco normalized to GAPDH using the $2^{-\Delta\Delta C_t}$ method and presented as fold-change relative to the third instar larvae using the $2^{-\Delta C_t}$ method. Data are represented as mean ± SD (n = 9). Cycle thresholds above 35 were considered non-detected (nd). (B) Intrapuparial development of *C. hominivorax* at three (3d), six (6d), and eight days (8d) after pupation. A closer view of the head region (white square) reveals the fully developed sensory structures of the adult form present in the late pupal stage. (C) Semi-quantitative comparison between developmental Orco expression in *C. hominivorax* (Chom) and *C. megacephala* (Cmeg) species by RT-PCR. (D) Detection of ChomOrco in adult screwworm female (♀) and male (♂) tissues. For all RT-PCR assays: Amplifications were made in replicates (n = 3), including no template controls (ntc), and GAPDH was amplified as an internal control. Cropped images (delineated by black lines) are from samples run on different gels. Full-length gels are displayed in Supplementary Fig. S2. (E) The main olfactory structures of *C. hominivorax* viewed under electron microscopy. (panel a) Screwworm female’s head highlighting the antennae (ant), and the maxillary palps (mp). (panel b) A closer view of the proximal surface of the third antenna segment reveals a number of tricoide (tri), basiconic (bas), and two morphotypes of coelonic (coe) sensilla (panel c), named grooved (gro) and clavate (cla). These morphotypes are adorned with multi-wall pores (single arrows) and grooves (double arrows), which presumably facilitate the entrance of odor molecules into the antennae.
complex structures in adults\textsuperscript{30}, also suggesting that olfaction plays a major role in the adult life stage of the screwworm fly. A similar developmental expression of Orco was observed in the blowfly \textit{C. megacephala} (Fig. 2C) by semi-quantitative reverse transcription PCR (RT-PCR), suggesting that the regulation of Orco expression is conserved in closely related blowflies.

In insects, Orco is expressed in nearly all of the olfactory sensory neurons (OSNs)\textsuperscript{30}. Therefore, it was foreseen that \textit{ChomOrco} would be mainly detected in olfactory-related tissues of \textit{C. hominivorax} adults. Indeed, semi-quantitative amplifications revealed that \textit{ChomOrco} is highly expressed in the main olfactory appendages of both sexes in the screwworm fly, including the antennae and the maxillary palps (Fig. 2D). Screwworm's antennae are subdivided into three segments: the scape, pedicel and funiculus. The former accommodates a thin and plumose arista, as seen in Fig. 2E (panel \textit{a}). Scanning electron microscopy (SEM; Supplementary Methods) of the female's head revealed that \textit{C. hominivorax} funiculus is predominantly adorned by three classes of sensilla: coelonic, tricoide and basiconic (Fig. 2E; panel \textit{b}). A close view of the proximal portion of this segment exposed a number of other two morphotypes of coelonic sensilla, named grooved and clave\textsuperscript{41}, lying in deep bristle pits (Fig. 2E; panel \textit{c}). These subclasses of sensilla are characterized by the presence of grooves and multiple wall pores, which presumably allows the entry of molecules able to stimulate the chemoreception system located inside the antennae. Thus, the high level of \textit{ChomOrco} transcription in the antennae is correlated to the morphology of this appendage. In addition to classic olfactory organs, a considerable abundance of \textit{Orco} transcripts was also detected in legs and abdomen of both sexes, and in the female ovipositor (Fig. 2D), implying that these tissues may also have chemosensory roles in \textit{C. hominivorax}.

**CRISPR/Cas9 gene editing efficiently generated \textit{ChomOrco} null germline mutants.** The evolutionary conservation of the \textit{Orco} gene among dipterans might be translated into a conserved molecular function, suggesting that \textit{ChomOrco} is required for normal OR-mediated olfaction in \textit{C. hominivorax}. To test this assumption, we knocked out \textit{ChomOrco} using CRISPR/Cas9 genome editing protocols we recently optimized for screwworm\textsuperscript{23}. Initially, we tested in vivo two single guide RNAs (sgRNAs), targeting exons 1 (sgR-\textit{Orco-E}1) and 2b (sgR-\textit{Orco-E}2b) of the \textit{ChomOrco} gene (Fig. 3A). Microinjections of pre-assembled ribonucleoproteins (RNPs), supplemented with a fluorescent protein-expressing plasmid, were performed in a small number of screwworm embryos (\(n \approx 100\)). Hatching fluorescent larvae (Supplementary Fig. S3) were examined for the presence of indels by T7 endonuclease 1 assays (T7E1), and induced genome modifications were quantified by Illumina sequencing (Supplementary Methods). Although both designed sgRNAs were found active, sgR-\textit{Orco-E}1 outperformed sgR-\textit{Orco-E}2b in induced mutagenesis (Fig. 3B,C). Indeed, microinjections with multiplexed sgRNAs poorly generated large deletion events between the targeted regions (Supplementary Fig. S4), most likely due to efficiency differences between designed sgRNAs.

Based on these initial results, new microinjections were carried out with sgR-\textit{Orco-E}1 (\(n = 750\)). Out of the 329 hatching larvae (larvae surviving rate: 44\%), a total of 154 showed expression of the co-injected fluorescent protein marker gene (putative microinjection success: 47\%). Marked larvae were collected and reared until adulthood, and 28 healthy adults were obtained (adult surviving rate: 18\%), including 12 males and 16 females. Only surviving G\(_0\) males were kept for backcrossing, as a high level of sterility has been previously observed for females developed from microinjections\textsuperscript{23}. Non-lethal DNA extractions (Supplementary Methods and Supplementary Fig. S6) were used as templates for T7E1 genotyping, which revealed that all selected males harbored indels at the \textit{ChomOrco} loci. Figure 3D summarizes the crossings and genotyping results obtained from this point. Ten males were randomly selected and individually backcrossed to virgin wild-type (wt) females to examine their founder capabilities. From each crossing, eight male-offspring (on average) were randomly sampled and genotyped as before. Out of the ten selected G\(_0\) putative founders, nine produced heterozygous G\(_1\) offspring (genotype\(\times\)wt ratio of genotypes (\(X^2 = 0.4, p = 0.52, d.f. = 1\)). Genotypes were confirmed by sequencing (Fig. 3E), and homozygous mutants inbred to establish a strain at G\(_4\). The \textit{ChomOrco}\textsuperscript{\textit{16}} mutants were evaluated for the loss of \textit{Orco} protein expression by immunostaining. Polyclonal antibody IC327 against the third intracellular loop of \textit{Orco} labeled the OSNs cell body and dendrites inside the sensilla of wt flies' antennae (Fig. 3F, \textit{above}), while no labeling was detected in mutant flies (Fig. 3F, \textit{below}). As in control slides (Supplementary Fig. S9), antennae sections of \textit{ChomOrco}\textsuperscript{\textit{16}} individuals lack detectable levels of Orco protein, confirming their knockout genotype.

**Disruption of \textit{Orco} impairs foraging and host-seeking behaviors in \textit{C. hominivorax}**. Homozygous \textit{ChomOrco}\textsuperscript{\textit{16}} mutants do not exhibit any visible phenotype or locomotion disabilities, and they are fertile. However, we observed a clear reduction in overall fitness relative to the wt flies. The knockout strain was weak and difficult to rear in culture. Although these characteristics make screwworm \textit{Orco} mutants undesirable for long-term rearing—for instance, the \textit{ChomOrco}\textsuperscript{\textit{16}} colony was maintained for eight inbreeding generations before col-
Figure 3. Knockout of *ChomOrco* by CRISPR/Cas9 induce complete loss of Orco protein in screwworm. (A) Schematic of the Cas9-targeted regions of *ChomOrco* and sgRNAs tested. Targeted sites are indicated by a scissor, PAM motif in red, and genotyping primers as blue arrows. Band migration patterns expected after T7E1 and dual-targeting assays are indicated below. (B,C) In vivo evaluation of sgR-Orco-E1 and sgR-Orco-E2b, respectively. A small number of surviving larvae (*n* = 6) were collected and genotyped by T7E1 (leftmost). PCR amplifications spanning targeted sites were pooled, Illumina sequenced, and genome modifications quantified using CRISPResso (rightmost chart). A diversity of allele variations is shown in Supplementary Fig. S3. Cropped images (delineated by black lines) of PCRs and T7E1 experiments are from samples run on the same gels (one for each experiment). Full-length gels are displayed in Supplementary Fig. S5. wt = wildtype sample; NTC = no template control; Ldd = 100 bp DNA Ladder (NEB). (D) Crossing scheme used to develop Orco mutant strains. Briefly, G0 flies were genotyped by T7E1, and mosaic males (Orco**−/**+) backcrossed to wt females (Orco**+/+**). Heterozygous individuals (Orco**+/-** at G1 were genotyped by sequencing, and siblings harboring a −16 bp mutation inbred at G2. Heterozygous (Orco**+/-**) and homozygous (Orco**−/−**) flies at G3 were identified by in vitro Cas9-antibody (Supplementary Fig. S8) and inbred to establish the heterozygous (OR16ko) and homozygous (*ChomOrco*16) strains. (E) Sequencing confirmation of the mutant genotypes in comparison with the wt allele. (F) Immunostaining of antennae sections from wt (above) and *ChomOrco*16 (KO, below) flies, showing cell nuclei (DAPI) and Orco protein (OR83b-IC3) localization within cell body (white arrows) and dendrites (black arrows) of screwworm’ OSNs. As in control slides (Supplementary Fig. S9), mutant flies display undetectable levels of Orco protein.
drastically reduced attraction to the oviposition media (Wilcoxon test against wt: $W = 80, p < 0.001$), exhibiting a lack of decision-making and impaired flight orientation towards the stimuli source (Fig. 4D and Supplementary Movie S1). Comparable behaviors were observed for "antennless" females. These outcomes demonstrate that $Orco$ is required for normal host-seeking behavior in $C. hominivorax$ and suggest that the perception of wound-derived odors by screwworm females relies, at least in part, on OR-mediated olfaction.

**Discussion**

The emergence of the obligate ectoparasitic lifestyle in blowflies is a fascinating biological problem, which has been challenging scientists for over a century. Surprisingly, few studies attempted to uncover the genetic basis of adaptive traits in this diverse group of flies. Since blowflies rely on odors to seek for oviposition sites, we hypothesized that changes in the olfactory system played a central role in the evolution of a free-living to a parasitic habit within the group. We adopted $C. hominivorax$ as a model to expand our knowledge on the olfactory-driven behaviors in parasitic blowflies and focused our research on the characterization of the $Orco$ gene, a required companion of all ORs. The $ChomOrco$ is highly conserved within Diptera (Fig. 1A, Supplementary Fig. S1, and Supplementary Table S1), as a reflection of a strong purifying selection regime (Fig. 1C). Same signatures of sequence evolution were found in the distantly related species $D. suzukii$ (Fig. 1C), an invasive phytophagous fruit fly. These observations indicate that other more divergent genes, rather than $Orco$, are responsible for the unique olfactory landscape of $C. hominivorax$. In fact, the functional conservation of $Orco$ across ca.
290 Ma of insect evolution was illustrated by Jones et al.45. By using transgenic constructs, the authors demonstrated the feasibility of using Orco orthologs from close and distantly related species to rescue functional defects in Orco mutants of Drosophila, clearly demonstrating the critical importance of this gene for insect olfaction.

Nevertheless, the evaluation of ChomOrco expression allows us to speculate on the contribution of olfaction to many aspects of screwworm biology. Quantitative analysis indicated that the olfactory system changes drastically throughout development in C. hominivorax (Fig. 2A). Orco transcription appears at the last hours of embryogenesis, persisting during the first larval stage. Screwworm larvae survival depends on proper host selection made by the mothers, which prefer to lay eggs on dry borders of animal wounds and bodily orifices46. This decision prevents the embryos from drowning in body fluids while ensuring hatching larvae immediate access to a nutrient-rich environment. Newborn larvae might use olfactory cues to guide their way from the oviposition site into the substrate to feed. Once the feeding source is found, this olfactory-based orientation might be gradually replaced by contact chemoreception, such as gustatory, explaining the decrease in ChomOrco expression during subsequent larval stages (Fig. 2A). A similar pattern is observed in other blowflies, such as Lucilia sericata49 and C. megacephala (Fig. 2C), indicating that the modulation of Orco expression is also evolutionary conserved. Interestingly, food ingestion by D. melanogaster is enhanced in the presence of microorganism-derived odors48. Thus, a reduction in olfactory input might be related to the lower rates of larval survival observed for the Orco mutant strain developed in this study.

The relatively low expression of Orco during larval stages also reflects their morphologically simple olfactory system50. In fact, ChomOrco expression appears to be in synchrony with the morphogenesis of the adult peripheral olfactory system (Fig. 2A,B). The correlation between structure and function, in this case, is illustrated by the high expression of Orco in the antennae and maxillary palps of both sexes (Fig. 2D,E). Olfaction is indispensable for C. hominivorax adulthood, in particular to locate susceptible animals for oviposition42,51. Animal host-finding and selection by screwworms can be seen as three scalable steps, from initial activation to orientation, and landing, culminating in the final decision of egg-laying. As gravid screwworms approach putative animal hosts, visual cues encourage the selection of landing sites, and other systems such as tactile, gustatory, and thermal contribute to generating enough stimuli for the egg-laying decision51. As in other Calyptratae flies, Orco transcription is detected in screwworm legs and abdomen of both sexes, and in the female ovipositor (Fig. 2D), suggesting that ChomOrco has a broader chemosensory role in C. hominivorax. Additionally, these appendages are likely to harbor unknown olfactory structures in the screwworm, as widely described in other species50,54–56, which in females would enhance the acquisition of short-range and contact stimuli required for oviposition1,45,52. Indeed, we noticed that Orco mutants invest considerably more time examining oviposition devices in comparison to wt flies, often laying smaller batches of eggs, presumably due to their chemoreception disabilities.

Odors emitted from wounded animals are perceived by gravid screwworm females through receptors located in the antennae, which are responsible for eliciting flight orientation towards putative animal hosts8,19,32,95. This was first demonstrated by Devaney et al.52, which found that the removal of screwworm females’ antennae is enough to disrupt their host-seeking behavior. This finding was subsequently confirmed by olfactometer assays53, and corroborated by the characterization of screwworm’s antennae ultrastructures51. Here, we present a further step in dissecting the behavioral responses mediated through olfactory receptor families in screwworms, by developing a germline Orco null strain of C. hominivorax using CRISPR/Cas9 genome editing (Fig. 3). Without Orco, the correct localization of all ORs is compromised, preventing the OR-signaling while maintaining other chemosensory pathways unharmed53,29,30,66. This knockout system allowed us to evaluate the contribution of OR and IR olfactory receptor families on C. hominivorax foraging and host-seeking behaviors using two-choice trap assays (Fig. 4A,B). While wt and heterozygous flies are highly attracted to both honey and oviposition devices, the disruption of Orco in screwworm resulted in a lack of decision-making and impaired flight-orientation, suggesting they were unable to perceive either smell (Fig. 4C,D, and Supplementary Movie S1). Furthermore, experiments with ablated individuals revealed that other chemosensory appendages do not compensate for the absence of the antennae to elicit flight-orientation towards these odors. The consequences of Orco disruption on specific traits have been described in a number of non-classical model insects, including social behavior in ants34, conspecific recognition in locust8,49, foraging and mating in moths33,35,36, human host-seeking in mosquito52, and oviposition in the spotted wing Drosophila17. Together, these studies unlock the major role of the OR gene family on insect evolution. Likewise, our results demonstrate that OR-mediated olfaction is required, at least in part, for normal screwworm host-seeking behavior, by inducing flight-orientation towards animal host-derived odors. They further illustrate one of many potential roles that Orco and ORs play in the ectoparasitism habit of the screwworm fly. Consequently, changes in the OR-olfactory pathway are expected to have contributed to shaping the ecological niche preference in the C. hominivorax lineage during blowfly evolution.

In the past decade, there has been great progress in the development of genomic tools supporting investigations on screwworm biology, including transcriptome sequencing60–62, molecular tools enabling gene silencing, editing and engineering12,63,64, and more recently the first reference genome assembly17. Together with this increasing availability of genomic resources, the results presented here set the stage for a broader investigation focusing on the genetics underlying specific traits in screwworm. It will be possible, for example, to search for signatures of diversifying selection on chemosensory gene families and evaluate their expression profiles at contrasting C. hominivorax physiological states. Supporting this idea, a recent study65 found that screwworm females and males exhibit very distinct antennal expression profiles, including many genes encoding for ORs. The study shows that at least one OR displaying a female-biased expression might have a role in the screwworm oviposition behavior. The ChomOr19 gene, as named by the authors, is an ortholog to the DmelOr7a; an OR related to aggregation and egg-laying decision in D. melanogaster66. Functional analysis of ChomOr19 and other genes of interest will benefit from our CRISPR/Cas9 methods developed for screwworm, allowing us to investigate their contribution to the different life strategies adopted by blowflies. From an applied perspective,
these efforts may assist in the identification of novel targets for alternative genetic strategies aiming to interfere with specific screwworm behaviors, in addition to the discovery of new environmentally safe semiochemicals to be used in integrated pest management programs. We have just begun a deep dive into the genetic basis of niche specialization in C. hominivorax, a species that is on its way to becoming a compelling model organism to study the link between chemoperception evolution and the emergence of novel ecological adaptations in the Calliphoridae family.

Methods
Flies. The C. hominivorax wt strain J06 was used in this study. The strain is routinely reared in the Agricultural Research Service (ARS) laboratory, located inside the Commission for the Eradication and Prevention of Screwworms (COPEG) biosecurity plant in Panama, under conditions previously described. Samples of Cochliomyia macellaria and C. megacephala were obtained from the frozen tissue collection of the Department of Genetics, Evolution, Microbiology and Immunology at the University of Campinas (UNICAMP), Brazil.

RNA and cDNA. Specimens were collected from colonies, while specific tissues were dissected from 3-days-old adult flies fasted for 12 h. Samples were rinsed with 0.1% DEPC-treated water and immediately homogenized in TRIzol reagent (Invitrogen). Total RNA was isolated according to manufacturer’s instructions. All extractions were DNase-treated in a 20 µl reaction containing 4 U of TURBO DNase (Invitrogen), 20 U of Ribolock RNase Inhibitor (Thermo Scientific), 1X Reaction Buffer and 10 µg of total RNA. Treatments were performed at 37 °C for 30 min and stopped by the addition of 15 mM of EDTA (pH 8.0) followed by an incubation at 75 °C for 10 min. To ensure complete digestion of genomic DNA, a total of 1 µg of each RNA preparation was visualized in a 1% denaturing agarose gel (1X MOPS, 2% formaldehyde) post-stained with Ethidium Bromide (EtBr; 0.5 µg/ml). Samples were previously mixed with 2X volume of Gel Loading Buffer II (Invitrogen), denatured at 65 °C for 20 min, and let cool down in ice for 5 min before gel applications. First-strand cDNAs were synthesized from 2 µg of DNase-treated RNA using the SuperScript II (Invitrogen) protocol with the Oligo(dT)12–18 primer (Invitrogen). Reverse transcriptions were performed at 42 °C for 1 h, terminated at 70 °C for 15 min, and stored at −20 °C.

RACE and genomic region. Specification for all primers used in this study can be found in Supplementary Table S4. First strand cDNA was used as template for the amplification of a 660 bp region of Orco transcripts from C. hominivorax (ChomOrco) and C. macellaria (CmacOrco; used as a control in this study) in a 50 µl PCR reaction containing 0.2 µM of each Orco-F1 and Orco-R1 primers, 80 µM of dNTPs, 10% Bovine Serum Albumin (BSA at 5 mg/ml), 1X Taq buffer supplied with 1.5 mM of MgCl₂ and 1.25 U of recombinant Taq DNA polymerase (Invitrogen). Amplifications were carried out in the following conditions: 95 °C for 3 min, 35 cycles of [95 °C for 30 s, 60 °C for 45 s and 72 °C for 60 s], and a final extension at 72 °C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), directly TA cloned into a pGEM-T Easy Vector (Promega) and sequenced in a 3730xl DNA Analyzer (Applied Biosystems) using the universal primers from 2 µg of DNase-treated mRNA using the SuperScript II (Invitrogen) protocol and the Oligo(dT)12–18 primer (Invitrogen). Reverse transcriptions were performed at 42 °C for 1 h, terminated at 70 °C for 15 min, and stored at −20 °C.

Evolutionary analysis. Selected Diptera Orco sequences (Supplementary Table S3) were codon-aligned using the Muscle algorithm implemented in MEGA X. Neighbor-Joining (NJ) method was used to estimate uncorrected distances (p-distance; Supplementary Table S1). Maximum-likelihood (ML) reconstructions were made in RAxML v.8 using the PROTGAMMA model and JTT substitution matrix for the amino acid alignment. Node supports were assessed by 500 non-parametric bootstrap replicates. The clade Aedes aegypti + Anopholes gambiace was defined as the outgroup. Adaptive evolution was tested using CodEM implemented in PAML4. Normalized non-synonymous (dN) to synonymous (dS) substitution rates (ω) were estimated using branch-site models to detect events of episodic selection on amino acid sites at specific lineages (foreground branches). Likelihood ratio tests (LRTs) were performed between the alternative model bsa (positive selection) and the null model bsa1 (neutral). Significance of LRT results were determined by chi-squared (χ²) tests, and Bayes Empirical Bayes analysis (BEB) was used to infer amino acid sites under selection regime ([cutoff ≥ 0.95]. Consensus locations of transmembrane domains (TM) within ChomOrco were predicted by TOPCONS, and significantly characterized sites were mapped onto the predicted protein topology as modeled in Proter77.

Expression. Evaluation of ChomOrco expression was conducted by Quantitative Real-Time PCR (qPCR). First-strand fivefold diluted cDNAs (equivalent to 50 ng of DNase-treated RNA template, as determined by 1:5 serial dilution standard curve analysis) were used as templates in 12.5 µl amplification reactions containing 6.25 µl of SYBR Green PCR Master Mix (Applied Biosystems), and 0.4 µM of each Orco-F2 and Orco-R2 primer (Supplementary Table S4). Runs were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the following protocol: hold at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of [95 °C for 15 s and 62 °C for 60 s]. Amplification efficiency was evaluated by standard curve method, and melting curves were assessed to ensure unique product amplification (Supplementary Fig. S11). Data were analyzed with
Biological (\( n = 3 \)) and technical (\( n = 3 \)) replicates were performed under controlled conditions (25 ± 2 °C, 65 ± 5% RH, 12:12 L:D) during the morning (from 7 to 11 am), as favored by screwworm adult43. Testing cages were positioned below fluorescent lights and side-enfolded with white paper sheets to provide a homogeneous light distribution.

For multiplexed experiments, RNPs were pre-assembled separately and 1:1 mixed prior to the microinjections. The plasmid pBJ[Chsp83-ZsGreen]82 was added to the final injection cocktail (300 ng/µl) with specific sgRNA (200 ng/µl) in a Sodium Phosphate Buffer (supplied with 300 mM of KCl) at 37 °C for 10 min. Cropping, contrast and light corrections were made in the same software.

CRISPR/Cas9. CRISPR experiments were performed as previously described23. Single guide RNAs (sgRNAs) were designed by examining ChomOrco exons for the presence of protoscaler-adjacent motifs (PAMs, sequence NGG-3; where “N” is any base) using the standalone version of CRISPOR tool9 in the context of C. hominivorax genome (GenBank: GCA_004302925.1). The sgRNAs were synthesized as described by Bassett and Liu44, with minor modifications23, while purified recombinant Cas9 protein was obtained commercially (PNA Bio). Ribonucleoprotein complexes (RNPs) were pre-assembled by incubating Cas9 protein (500 ng/µl) with specific sgRNA (200 ng/µl) in a Sodium Phosphate Buffer (supplied with 300 mM of KCl) at 37 °C for 10 min.

Antennae were dissected from females and directly frozen in O.C.T. Compound (Sakura Tissue-Tek) at −20 °C. Cryosections were made in a Leica CM1850 at a thickness of 18 µm, and thaw-mounted on gelatin-coated microscope slides. Slides were air-dried at room temperature for 10 min, submersed in phosphate-buffered saline (PBS) supplemented with 0.05% Azide for 5 min, and blocked in PBS with 0.2% Triton X and 3% Bovine Serum Albumin (PBSTB) for 1 h. Sections were incubated with anti-Or83b (peptide HWYDG-SEEAKT, described in27) rabbit polyclonal antibody (1:100 in PBSTB) overnight at 4 °C in a humid chamber. Slides were washed for a total of 15 min in PBST (5 min per wash) and incubated at room temperature for 1 h with donkey anti-rabbit Alexa 488 secondary antibody (Life Technologies, diluted 1:500 in PBSTB). Slides were mounted with the Vectashield medium (Vector Labs), and sealed with nail polish. Stained antennae sections were imaged on a Leica TCS SP5 II confocal microscopy at the Life Sciences Core Facility (LaCTAD), located at UNICAMP. The wt and mutant samples were mounted in the same slides and imaged under the same settings. No signal was detected in the absence of primary or secondary antibodies (Supplementary Fig. S9).

Behavior. For the foraging assays, unmated adult flies of 3-to-4-day-old were fasted for 12 h with access to water, transferred without anesthesia to the test arena (BioQuip Inc., model 1450B), and left to acclimate for 15 min before the trials. Groups of 15 to 25 flies (males to females, ratio 1:1) were tested in each trial. Odor bait consisted of 400 mg of natural honey (handcrafted at Holambra-SP, Brazil) applied on a 25 mm Whatman filter paper (GE Healthcare), while the control bait contained the same weight of glycerol (Sigma-Aldrich). Two-choice assays were performed under controlled conditions (25 ± 2 °C, 65 ± 5% RH, 12:12 L:D) during the morning (from 7 to 11 am), as favored by screwworm adult43. Testing cages were positioned below fluorescent lights and side-enfolded with white paper sheets to provide a homogeneous light distribution from above. Control trials ensured that flies did not prefer one side of the cage upon the other (Supplementary Fig. S10). Ablated “antennal-less” flies (i.e., adults that had at least the 3rd antennae segment physically removed before the day trials) were also tested in order to ensure that choices were guided by olfaction. For the oviposition assays, groups of 10 to 20 fed and post-mated adult females of 6-to-9-day-old were tested in each trial. Odor bait contained 10 ml of 25% warmed waste larval rearing media67, while the same volume of warmed distilled water was used as control. Oviposition assays were performed in complete darkness, as it appears to increase oviposition attraction45 while also avoiding visual cues. Traps (Fig. 4B, see Supplementary Methods for craft details) were alternatively placed in opposite sides of cages, and their positions altered for every trial. Captures were scored after 4 h (foraging) or 90 min (oviposition). Attraction index was calculated as: \( AI = (n_{odor} - n_{control}) / n_{control} \).
n_{total}, where n_{odor} is the number of flies captured in the odorant trap, n_{control} the number of flies captured in the control trap, and n_{total} the total of flies tested. The AI ranges from −1 (complete avoidance) to +1 (complete attraction). Values of zero characterizes a neutral or non-detection of odors. Significant deviations of AI were tested with the two-sample Wilcoxon rank-sum test with continuity correction using the wilcox.test function implemented in the R/stats package.

Data availability
The ChomOrco sequences were submitted to GenBank and can be retrieved under the accession numbers MT226797 and MT226799, for transcript and genomic sequences, respectively. The CmacOrco transcript can be retrieved under the GenBank accession number MT226798. A stable screwworm heterozygous population for ChomOrco (named Orco16Ko) is being maintained at the ARS laboratory at COPEG in Panama.

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**Author contributions**  
D.F.P., C.C. and A.C.M.J., conceptualized this study with insights from W.O.M., M.J.S. and A.M.L.A.-E. D.F.P., C.C., A.C.M.J., A.P.A., W.O.M. and M.J.S. conceived, designed, and/or performed the experiments, analyzed and/or interpreted the collected data. J.C. performed the antennae EM imaging with inputs from D.F.P. A.S.V. performed *Orco* immunostaining and imaging with inputs from D.F.P. S.R.S., A.A.P.-L., W.O.M., M.J.S., A.M.L.A.-E. provided the resources for this work. C.C. and A.M.L.A.-E. supervised and administered the study. D.F.P., C.C., A.C.M.J., A.A.P.-L., W.O.M., M.J.S. and A.M.L.A.-E. were engaged in the acquisition of the financial support for this study. D.F.P. wrote the manuscript with substantial insights from C.C., A.C.M.J., A.A.P.-L., M.J.S. and W.O.M. All authors approved the final version of the manuscript.

**Competing interests**  
The authors declare no competing interests.

**Additional information**  
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**Correspondence** and requests for materials should be addressed to C.C.

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