Courtship pheromones represent an important facet in the reproductive biology of many species throughout the Animal Kingdom. They are chemical signals, expressed by sexually mature males or females, that trigger a specific reaction in the opposite gender. Living amphibians (frogs, salamanders and caecilians) are widely acknowledged for their remarkable and well-documented diversity in reproductive strategies and sexual glands. In contrast however, only six molecules that serve a pheromone function have been characterized. Many aspects regarding the taxonomic diversity of amphibians that use courtship pheromones, the diversity of secreting tissues involved, and the molecular structural variation that constitutes a typical amphibian pheromone system remain unexplored. By combining various techniques including transcriptomics, proteomics, genomics, phylogenetics, CT scan imaging and behavioral experiments, this work reports on newly identified pheromone-secreting glands, the evolutionary diversification of previously identified pheromone systems, and a newly developed behavioral assay for future pheromone identification. In a first chapter, a well-known male-specific structure, nuptial pads, in one of the most common frog species of Europe, is identified as a previously overlooked pheromone-secreting organ. This finding not only broadens the range of known pheromone-producing glands but also extends the known phylogenetic range of pheromone-secreting amphibians. In the second chapter, an overview is provided of the extraordinary structural diversity in a widespread pheromone system in salamanders, named Sodefrin Precursor-like Factor (SPF). To understand how this pheromone system underpinned the evolution of courtship strategies, we screened the cloacal courtship gland of multiple species with distinct courtship behaviors. In the third chapter, our knowledge on SPF diversification is expanded by expression pattern comparisons of multiple courtship glands and other tissues in one species. Linking the retrieved SPF variation to the evolution of salamander mating strategies in a phylogenetic framework delivers unprecedented insights in the co-evolution of chemical communication and reproductive behavior. All of these chapters elucidate previously unknown patterns of diversity at different levels of biological organisation.

Studies that have effectively characterized pheromone candidates with a reliable bioassay are rare, partly due to the difficulty of designing an appropriate pheromone test. Often one species is selected as a model organism, and a behavioral test is developed to alter behavioral responses to pheromones of that particular species. However, when aiming at understanding pheromone evolution, it is interesting to investigate pheromone communication over a series of closely and more distantly related species, and to reconstruct evolutionary changes in species-specific chemical courtship signals. In Chapter Four, a test is presented that provides such possibility: the recognition of female cloacal gaping as a response to male secretions provides a relatively simple test that may be applied in the behavioral study of chemical communication across a wide diversity of species.

In conclusion, by focusing on specific aspects of amphibian courtship, this thesis describes an extraordinary evolutionary flexibility of amphibian chemical communication.